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Patent Docket P1101P2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Avi J. Ashkenazi, et al. Serial No.: 09/396,710 Filed: September 15, 1999 For: Apo-2 Receptor Antibodies	Group Art Unit: 1647 Examiner: Kaufman, C. **Customer No:09157**

RULE 131 DECLARATION

I, Avi J. Ashkenazi, hereby declare as follows:

1. I am the named inventor of the claimed subject matter of the above-identified patent application.

2. The above-identified patent application claims priority to application serial no. 08/857,216 filed with the Patent Office on May 15, 1997, and I am the named inventor in that priority application. A copy of my priority application serial no. 08/857,216 (hereinafter the "'216 application") is attached as Exhibit A.

3. All work described in the above-identified application and the '216 application was performed by me or on my behalf in the United States of America.

4. The '216 application filed on my behalf on May 15, 1997 demonstrates both my conception of the claimed invention of the present application and a constructive reduction to practice of the invention.

5. Experiments performed by me or on my behalf relating to the identification and structural characterization of the Apo-2 receptor are described, for example, in Example 1 of the '216 application, pages 58-62. In *in vitro* binding assays, I found that the Apo-2 receptor extracellular domain binds the ligand known as Apo-2 ligand (the '216 application, e.g., pages 63, lines 9-35 - page 64, lines 1-6). In further *in vitro* assays, I also found that the Apo-2 receptor was capable of inducing cell

death in transfected mammalian cells (the '216 application, page 64, lines 9-35 - page 65, lines 1 -13).

6. In the '216 application, agonist antibodies to the Apo-2 receptor are described. (See, e.g., Page 10, lines 3-5; Page 15, lines 7-10; Page 56, lines 21-23). More particularly, the '216 application discloses that an agonistic Apo-2 antibody may be employed to activate or stimulate apoptosis in mammalian cancer cells (Page 56, lines 21-23). Methods for making Apo-2 antibodies are described on pages 48-56 of the '216 application. Apoptotic activity in mammalian cells is described on, e.g., page 17, lines 1-12, of the '216 application.

7. The '216 application therefore demonstrates that agonist antibodies which bind Apo-2 receptor and stimulate apoptosis were conceived and constructively reduced to practice by the May 15, 1997 filing date of my patent application.

8. I have read and reviewed U.S. Patent Application Publication No. 2002/0160446 (corresponding to application serial number 09/811,088, hereinafter the "the '088 application" a copy of which is attached as Exhibit B) which was filed with the Patent Office on March 16, 2001. I understand that the '088 application is a continuation-in-part application of various earlier-filed applications, which include:

- U.S.S.N. 09/757,421 (filed Jan. 10, 2001), now abandoned, which claims priority from U.S.S.N. 08/843,652 (filed Apr. 16, 1997)("the '652 application"), now abandoned.

I have read and reviewed the '652 application. I note that in these applications, the disclosed and claimed receptor sequence is termed "Tango-63."

9. The '652 application discloses two polynucleotide sequences, "Tango-63d" and "Tango-63e" that encode a 440 and 411 amino acid sequence, respectively. See, e.g., page 4, lines 20-21. I will refer to these two sequences in this declaration as the "Tango-63" sequences. The '652 application postulates that Tango-63 sequences are similar to members of the TNF receptor superfamily. See, e.g., page 4, lines 17-19 and page 63, lines 1-3. The '652 application provides no disclosure of any particular sequence-based comparison (such as a sequence alignment) between the Tango-63 sequences and other members of the TNF receptor family. There is a general statement on page

58, lines 23-25 of the '652 application that there is "conservation" between the intracellular domains of TNFR-1 and Tango-63.

10. The '652 application states that "members of the TNF receptor superfamily are characterized by the presence of cysteine-rich repeats in their extracellular domains, and the Fas/APO-1 receptor and TNFR-1 also share an intracellular region of homology designated the "death domain"…. See, page 63, lines 4-8. The '652 application does not characterize any particular domains or motifs that may be present in the Tango-63 sequences themselves (e.g., any particular regions of the Tango-63 sequences that may constitute or act as an extracellular domain, intracellular domain or death domain are not described). Accordingly, there is no clear comparison made in the '652 application between the Tango-63 sequences to other known members of the TNF receptor family such as Fas or TNFR-1.

11. The '652 application likewise provides no analysis or data regarding the identity or conservation of specific amino acids within any putative death domain, which were known to be crucial for activity of the death domain of TNFR-1 (see, e.g., Table 2, Tartaglia et al., Cell, 74, 845-853 (1993); Fig. 4B, Brojatsch et al., Cell, 87, 845-855 (1996)).

12. I note that the functional complexity of TNF receptor superfamily members that contain death domain motifs was well known in the art at the time of the filing of the '652 application, particularly with respect to the biological functions associated with binding of ligands to such receptors. One example is the low affinity NGF receptor (p75 NGFR, also called "neurotrophin receptor" or "NTR") known prior to the filing date of the '652 application. In Rabizadeh et al., Science, 261, 345-348 (1993), the authors teach that "expression of p75 NGFR induced neural cell death constitutively when p75 NGFR was unbound; binding by NGF or monoclonal antibody, however, inhibited cell death induced by p75 NGFR". In Chapman, FEBS Lett., 374, 216-220 (1995), the author states that "Unlike TNFR-1 and Fas, cell death induced by NTR (namely p75 NGFR) is reversed rather than caused by ligand binding". Thus, at the time of the filing of the '652 application, binding of ligand to NTR was known to inhibit, rather than stimulate, apoptosis. Therefore, the mere presence of a death domain related sequence is not, standing alone, indicative of the specific function or functions of a receptor in the TNF receptor superfamily, particularly those functions associated with ligand binding to such receptor.

13. The '652 application fails to identify a ligand that specifically binds to the putative receptors encoded by the Tango-63 sequences. Based on my review of the '652 application, it is my belief that the applicant of the '652 application was not aware at the time the application was filed that the Tango-63 bound any specific ligand. See, e.g., page 7, lines 4-11 wherein it states that "...the polypeptides may function in a ligand-independent manner. In the event a ligand is identified, one could then determine whether that ligand acts as a full or partial agonist or antagonist of the polypeptide of the invention...".

14. The '652 application contains no disclosure pertaining to antibodies to the expression products of the Tango-63 sequences that have characteristics of the claimed antibodies of the present application. The disclosure of the '652 application simply provides a general indication that antibodies can be raised to the polypeptides of the invention (see, e.g., page 17, lines 22-24) and can be used in diagnostic or prognostic techniques or in screening assays for the evaluation of the effect of test compounds on expression and/or activity of Tango-63. See, e.g., '652 application at page 33, beginning at line 13 to page 36.

15. There is no description in the '652 application of Tango-63 receptor agonist antibodies which induce apoptosis in mammalian cells. The disclosure refers to "compounds" which may modulate the expression or activity of Tango-63, but such compounds described generally concern, e.g., small molecules, ribozymes, naturally-occurring or synthetic ligand, and anti-sense nucleic acid molecules. See, e.g., page 8, lines 15-26; page 9, lines 4-14; page 10, lines 9-21; page 18, lines 15-26 and page 60, lines 22-28. The '652 application does not describe or suggest such a compound to be an agonist antibody to the Tango-63 sequences. Therefore, it is my opinion that the '652 application neither discloses nor motivates one skilled in the art to make or use an anti-Tango-63 antibody as an agonist antibody that mimics ligand (i.e., apoptosis-inducing) activity.

16. The '652 application fails to identify or describe any examples of hybridomas or monoclonal antibodies that were actually produced against the Tango-63 sequences. There is likewise no description in the '652 application of any example of a monoclonal antibody that was actually produced which binds to Tango-63, or which has apoptosis-inducing, or ligand-mimicking, activity.

17. In light of the above facts and observations, it is my opinion that with respect to the '652 application one skilled in the art would find no suggestion to produce antibodies which bind to the putative receptors encoded by the Tango-63 sequences and which induce specific biological functions (e.g., apoptosis) upon binding to the putative receptor.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

3/22/04
Date

Avi J Ashkenazi
Avi J. Ashkenazi, Ph.D.

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Apo-2 Receptor

FIELD OF THE INVENTION

The present invention relates generally to the identification, isolation, and recombinant production of novel polypeptides, designated herein as "Apo-2".

BACKGROUND OF THE INVENTION

Apoptosis or "Programmed Cell Death"

Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, *e.g.*, Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of apoptotic cell death have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)]. Increased levels of apoptotic cell death may be associated with a variety of other pathological conditions, including AIDS, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic anemia, myocardial infarction, stroke, reperfusion injury, and toxin-induced liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, supra].

TNF Family of Cytokines

Various molecules, such as tumor necrosis factor-α ("TNF-α"), tumor necrosis factor-β ("TNF-β" or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996)]. Among these molecules, TNF-α, TNF-β, CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been reported to be involved in apoptotic cell death. Both TNF-α and TNF-β have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF-α is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory

Symposium on Programmed Cell Death, Abstr. No. 10, (1995)].

Mutations in the mouse Fas/Apo-1 receptor or ligand genes (called *lpr* and *gld*, respectively) have been associated with some autoimmune disorders, indicating that Apo-1 ligand may play a role in regulating the clonal deletion of self-reactive lymphocytes in the periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata et al., Science, 267:1449-1456 (1995)]. Apo-1 ligand is also reported to induce post-stimulation apoptosis in CD4-positive T lymphocytes and in B lymphocytes, and may be involved in the elimination of activated lymphocytes when their function is no longer needed [Krammer et al., supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies specifically binding to the Apo-1 receptor have been reported to exhibit cell killing activity that is comparable to or similar to that of TNF-α [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

TNF Family of Receptors

Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive polymorphisms have been associated with both TNF receptor genes [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions. The extracellular portions of both receptors are found naturally also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A.,

87:8331 (1990)]. More recently, the cloning of recombinant soluble TNF receptors was reported by Hale et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four cysteine-rich domains (CRDs) designated 1 through 4, starting from the NH_2-terminus. Each CRD is about 40 amino acids long and contains 4 to 6 cysteine residues at positions which are well conserved [Schall et al., supra; Loetscher et al., supra; Smith et al., supra; Nophar et al., supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2- amino acids from about 54 to about 97; CRD3- amino acids from about 98 to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about 55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4- amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435 (1993)]. The potential role of the CRDs in ligand binding is also described by Banner et al., supra.

A similar repetitive pattern of CRDs exists in several other cell-surface proteins, including the p75 nerve growth factor receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature, 325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J., 8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063 (1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al., supra]. CRDs are also found in the soluble TNFR (sTNFR)-like T2 proteins of the Shope and myxoma poxviruses [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335 (1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of these sequences indicates that the positions of the cysteine residues are well conserved. These receptors are sometimes collectively referred to as members of the TNF/NGF receptor superfamily. Recent studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in

this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF-α or anti-Apo-1 mouse monoclonal antibody [Krammer et al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 receptors may mediate cell killing through common signaling pathways [Id.].

The TNF family ligands identified to date, with the exception of lymphotoxin-α, are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, the receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF-α, Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the TNFR family have been identified. In Marsters et al., Curr. Biol., 6:750 (1996), investigators describe a full length native sequence human polypeptide, called Apo-3, which exhibits similarity to the TNFR

family in its extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain sequence [see also Marsters et al., Curr. Biol., 6:1669 (1996)]. Apo-3 has also been referred to by other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science, 274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al., Immunity, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4 was reported to contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

The Apoptosis-Inducing Signaling Complex

As presently understood, the cell death program contains at least three important elements - activators, inhibitors, and effectors; in *C. elegans*, these elements are encoded respectively by three genes, *Ced-4*, *Ced-9* and *Ced-3* [Steller, Science, 267:1445 (1995); Chinnaiyan et al., Science, 275:1122-1126 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell; 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF-κB [Tartaglia et al., Cell, 74:845-853 (1993); Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra; Nagata, Cell, 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the Drosophila protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, Cell, 81:479-482 (1995)]. Using the yeast-two hybrid system, Raven et al. report the identification of protein, wsl-1, which binds to the TNFR1 death domain [Raven et al., Programmed Cell Death Meeting, September 20-24, 1995, Abstract at page 127; Raven et al., European Cytokine Network, 7:Abstr. 82 at page 210 (April-June 1996)]. The

wsl-1 protein is described as being homologous to TNFR1 (48% identity) and having a restricted tissue distribution. According to Raven et al., the tissue distribution of wsl-1 is significantly different from the TNFR1 binding protein, TRADD.

Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signalling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., Cell, 81:505-512 (1995); Boldin et al., J. Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al., J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the *Ced-3*-related protease, MACHα/FLICE (caspase 8), into the death signalling complex [Boldin et al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)]. MACHα/FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1β converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, *ced-3*, and to the mammalian IL-1-converting enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, *crmA* [Ray et al., Cell, 69:597-604 (1992); Tewari et al., Cell, 81:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., Nature, 375:78-81 (1995); Tewari et al., J. Biol. Chem., 270:3255-3260 (1995)].

As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF-κB [Tewari et al., Curr. Op. Genet. Develop., 6:39-44 (1996)]. NF-κB is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., Genes Develop., 9:2723-2735

(1996); Baldwin, Ann. Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF-κB is complexed with members of the IκB inhibitor family; upon inactivation of the IκB in response to certain stimuli, released NF-κB translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode novel polypeptides, designated in the present application as "Apo-2." It is believed that Apo-2 is a member of the TNFR family; full-length native sequence human Apo-2 polypeptide exhibits some similarities to some known TNFRs, including a cytoplasmic death domain region. Full-length native sequence human Apo-2 also exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats. Apo-2 polypeptide has been found to be capable of triggering caspase-dependent apoptosis and activating NF-κB. Applicants surprisingly found that the soluble extracellular domain of Apo-2 binds Apo-2 ligand (Apo-2L) and can inhibit Apo-2 ligand function. It is presently believed that Apo-2 ligand can signal via at least two different receptors, DR4 and the newly described Apo-2 herein.

In one embodiment, the invention provides isolated Apo-2 polypeptide. In particular, the invention provides isolated native sequence Apo-2 polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). In other embodiments, the isolated Apo-2 polypeptide comprises at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated

death domain sequence of Apo-2. Optionally, the isolated death domain sequence comprises amino acid residues 324 to 391 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides chimeric molecules comprising Apo-2 polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-2 fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of Apo-2 fused to a heterologous polypeptide or amino acid sequence, such as an immunoglobulin sequence.

In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2 polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an Apo-2 polypeptide or a particular domain of Apo-2, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. In one embodiment, the nucleic acid sequence is selected from:

(a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 411 (i.e., nucleotides 140-142 through 1370-1372), inclusive;

(b) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 182 (i.e., nucleotides 140-142 through 683-685), inclusive;

(c) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 54 to residue 182 (i.e., nucleotides 299-301 through 683-685), inclusive;

(d) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 324 to residue 391 (i.e., nucleotides 1109-1111 through 1310-1312), inclusive; or

(e) a sequence corresponding to the sequence of (a), (b), (c) or (d) within the scope of degeneracy of the genetic code.

In a further embodiment, the invention provides a vector comprising the nucleic acid molecule encoding the Apo-2 polypeptide or particular domain of Apo-2. A host cell comprising the vector

or the nucleic acid molecule is also provided. A method of producing Apo-2 is further provided.

In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic or neutralizing antibody.

In another embodiment, the invention provides non-human, transgenic or knock-out animals.

A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of a native sequence human Apo-2 cDNA and its derived amino acid sequence.

Figure 2A shows the derived amino acid sequence of a native sequence human Apo-2 - the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined.

Figure 2B shows an alignment and comparison of the death domain sequences of native sequence human Apo-2, DR4, Apo-3/DR3, TNFR1, and Fas/Apo-1 (CD95). Asterisks indicate residues that are essential for death signaling by TNFR1 [Tartaglia et al., supra].

Figure 3 shows the interaction of the Apo-2 ECD with Apo-2L. Supernatants from mock-transfected 293 cells or from 293 cells transfected with Flag epitope-tagged Apo-2 ECD were incubated with poly-His-tagged Apo-2L and subjected to immunoprecipitation with anti-Flag conjugated or Nickel conjugated agarose beads. The precipitated proteins were resolved by electrophoresis on polyacrylamide gels, and detected by immunoblot with anti-Apo-2L or anti-Flag antibody.

Figure 4 shows the induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (A, B) or HeLa cells (C) were transfected by pRK5 vector or by pRK5-based plasmids encoding Apo-2 and/or CrmA. Apoptosis was assessed by morphology (A), DNA fragmentation (B), or by FACS (C-

E). Soluble Apo-2L was pre-incubated with buffer or affinity-purified Apo-2 ECD together with anti-Flag antibody or Apo-2 ECD immunoadhesin or DR4 or TNFR1 immunoadhesins and added to HeLa cells. The cells were later analyzed for apoptosis (D). Dose-response analysis using Apo-2L with Apo-2 ECD immunoadhesin was also determined (E).

Figure 5 shows activation of NF-κB by Apo-2, DR4, and Apo-2L. (A) HeLa cells were transfected with expression plasmids encoding the indicated proteins. Nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay. (B) HeLa cells or MCF7 cells were treated with buffer, Apo-2L or TNF-alpha and assayed for NF-κB activity. (C) HeLa cells were preincubated with buffer, ALLN or cyclohexamide before addition of Apo-2L. Apoptosis was later analyzed by FACS.

Figure 6 shows expression of Apo-2 mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The terms "Apo-2 polypeptide" and "Apo-2" when used herein encompass native sequence Apo-2 and Apo-2 variants (which are further defined herein). These terms encompass Apo-2 from a variety of mammals, including humans. The Apo-2 may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

A "native sequence Apo-2" comprises a polypeptide having the same amino acid sequence as an Apo-2 derived from nature. Thus, a native sequence Apo-2 can have the amino acid sequence of naturally-occurring Apo-2 from any mammal. Such native sequence Apo-2 can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2" specifically encompasses naturally-occurring truncated or secreted forms of the Apo-2 (*e.g.*, an extracellular domain sequence), naturally-occurring variant forms (*e.g.*, alternatively spliced forms) and naturally-

occurring allelic variants of the Apo-2. A naturally-occurring variant form of the Apo-2 includes an Apo-2 having an amino acid substitution at residue 410 in the amino acid sequence shown in Figure 1 (SEQ ID NO:1). In one embodiment of such naturally-ocurring variant form, the leucine residue at position 410 is substituted by a methionine residue. In Fig. 1 (SEQ ID NO:1), the amino acid residue at position 410 is identified as "Xaa" to indicate that the amino acid may, optionally, be either leucine or methionine. In Fig. 1 (SEQ ID NO:2), the nucleotide at position 1367 is identified as "W" to indicate that the nucleotide may be either adenine (A) or thymine (T) or uracil (U). In one embodiment of the invention, the native sequence Apo-2 is a mature or full-length native sequence Apo-2 comprising amino acids 1 to 411 of Fig. 1 (SEQ ID NO:1).

The "Apo-2 extracellular domain" or "Apo-2 ECD" refers to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally, Apo-2 ECD will comprise amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1) or amino acid residues 1 to 182 of Fig. 1 (SEQ ID NO:1).

"Apo-2 variant" means a biologically active Apo-2 as defined below having at least about 80% amino acid sequence identity with the Apo-2 having the deduced amino acid sequence shown in Fig. 1 (SEQ ID NO:1) for a full-length native sequence human Apo-2. Such Apo-2 variants include, for instance, Apo-2 polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the sequence of Fig. 1 (SEQ ID NO:1). Ordinarily, an Apo-2 variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the amino acid sequence of Fig. 1 (SEQ ID NO:1).

"Percent (%) amino acid sequence identity" with respect

to the Apo-2 sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-2 sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN™ or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising Apo-2, or a domain sequence thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the Apo-2. The tag polypeptide preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning

cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the Apo-2 natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" Apo-2 nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the Apo-2 nucleic acid. An isolated Apo-2 nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 nucleic acid molecules therefore are distinguished from the Apo-2 nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 nucleic acid molecule includes Apo-2 nucleic acid molecules contained in cells that ordinarily express Apo-2 where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that

the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers single anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti-Apo-2 antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain (*e.g.* "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (*e.g.*, Fab, $F(ab')_2$, and Fv), so long as they exhibit the desired biological activity. See, *e.g.* U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous

population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Humanized" forms of non-human (*e.g.* murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

"Biologically active" and "desired biological activity" for the purposes herein mean having the ability to modulate

apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell *in vivo* or *ex vivo*.

The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

II. Compositions and Methods of the Invention

The present invention provides newly identified and isolated Apo-2 polypeptides. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides are described in further detail in the Examples below. Based upon the properties and characteristics of the Apo-2 polypeptides disclosed herein, it is Applicants' present belief that Apo-2 is a member of the TNFR family.

A description follows as to how Apo-2, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared.

A. Preparation of Apo-2

The description below relates primarily to production of Apo-2 by culturing cells transformed or transfected with a vector containing Apo-2 nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-2.

1. Isolation of DNA Encoding Apo-2

The DNA encoding Apo-2 may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 mRNA and to express it at a detectable level. Accordingly, human Apo-2 DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage libraries of human pancreas and kidney cDNA described in Example 1. The Apo-2-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-2 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer:A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

A preferred method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various human tissues. Example 1 below describes techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like ^{32}P-labeled ATP, biotinylation or enzyme labeling. Hybridization conditions,

inlcuding moderate stringency and high stringency, are provided in Sambrook et al., supra.

Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Apo-2 variants can be prepared by introducing appropriate nucleotide changes into the Apo-2 DNA, or by synthesis of the desired Apo-2 polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the Apo-2, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

Variations in the native full-length sequence Apo-2 or in various domains of the Apo-2 described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the Apo-2 that results in a change in the amino acid sequence of the Apo-2 as compared with the native sequence Apo-2. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the Apo-2 molecule. The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the Apo-2 variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence which are involved in the interaction with a particular ligand or receptor. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is the preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Once selected Apo-2 variants are produced, they can be contacted with, for instance, Apo-2L, and the interaction, if any, can be determined. The interaction between the Apo-2 variant and Apo-2L can be measured by an *in vitro* assay, such as described in the Examples below. While any number of analytical measurments can be used to compare activities and properties between a native sequence Apo-2 and an Apo-2 variant, a convenient one for binding is the dissociation constant K_d of the complex formed between the Apo-2 variant and Apo-2L as compared to the K_d for the native sequence Apo-2. Generally, a $\geq$ 3-fold increase or decrease in K_d per substituted residue indicates that the substituted residue(s) is active in the interaction of the native sequence Apo-2 with the Apo-2L.

Optionally, representative sites in the Apo-2 sequence suitable for mutagenesis would include sites within the extracellular domain, and particularly, within one or both of the cysteine-rich domains. Such variations can be accomplished using the methods described above.

2. Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding Apo-2 may be inserted into a replicable vector for further cloning

(amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

(i) Signal Sequence Component

The Apo-2 may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, *e.g.*, the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2 in the cell membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

The DNA for such precursor region is preferably ligated in reading frame to DNA encoding Apo-2.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

Most expression vectors are "shuttle" vectors, *i.e.*, they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of Apo-2 DNA. However, the recovery of genomic DNA encoding Apo-2 is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Apo-2 DNA.

(iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host

cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)] or hygromycin [Sugden et al., Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the Apo-2 nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes Apo-2. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of Apo-2 are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, adenosine deaminase, and

ornithine decarboxylase.

Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding Apo-2. This amplification technique can be used with any otherwise suitable host, *e.g.*, ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding Apo-2, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.*, kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)]. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., Curr. Genet., 12:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, Bio/Technology, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed [Fleer et al., Bio/Technology, 9:968-975 (1991)].

(iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the Apo-2 nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, *e.g.*, the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2 promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 DNA.

Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-

25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding Apo-2 [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-2.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Apo-2 transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the Apo-2 sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon β1 gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter].

(v) Enhancer Element Component

Transcription of a DNA encoding the Apo-2 of this

invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983)] to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the Apo-2 coding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding Apo-2.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the

plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology, 65:499 (1980).

(viii) Transient Expression Vectors

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying Apo-2 variants.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or

Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia, e.g., E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., Salmonella typhimurium, Serratia, e.g., Serratia marcescans,* and *Shigella,* as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g., B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa,* and *Streptomyces.* Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2-encoding vectors. *Saccharomyces cerevisiae,* or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

Suitable host cells for the expression of glycosylated Apo-2 are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, *e.g.,* Luckow et al., Bio/Technology, 6:47-55 (1988); Miller et al., in Genetic Engineering, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., Nature, 315:592-594 (1985)]. A variety of viral strains for transfection are publicly available, *e.g.,* the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens.* During incubation of the plant cell culture with *A. tumefaciens,* the DNA encoding the Apo-2

can be transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Apo-2-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences [Depicker et al., J. Mol. Appl. Gen., 1:561 (1982)]. In addition, DNA segments isolated from the upstream region of the T-DNA *780* gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue [EP 321,196 published 21 June 1989].

Propagation of vertebrate cells in culture (tissue culture) is also well known in the art [See, *e.g.*, Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in

fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

4. Culturing the Host Cells

Prokaryotic cells used to produce Apo-2 may be cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce Apo-2 may be

cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively,

antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, or luminescent labels.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence Apo-2 polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-2 DNA and encoding a specific antibody epitope.

6. Purification of Apo-2 Polypeptide

Forms of Apo-2 may be recovered from culture medium or from host cell lysates. If the Apo-2 is membrane-bound, it can be released from the membrane using a suitable detergent solution (*e.g.* Triton-X 100) or its extracellular domain may be released by enzymatic cleavage.

When Apo-2 is produced in a recombinant cell other than one of human origin, the Apo-2 is free of proteins or polypeptides of human origin. However, it may be desired to purify Apo-2 from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. Apo-2 thereafter is purified from

contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Apo-2 variants in which residues have been deleted, inserted, or substituted can be recovered in the same fashion as native sequence Apo-2, taking account of changes in properties occasioned by the variation. For example, preparation of an Apo-2 fusion with another protein or polypeptide, *e.g.*, a bacterial or viral antigen, immunoglobulin sequence, or receptor sequence, may facilitate purification; an immunoaffinity column containing antibody to the sequence can be used to adsorb the fusion polypeptide. Other types of affinity matrices also can be used.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native sequence Apo-2 may require modification to account for changes in the character of Apo-2 or its variants upon expression in recombinant cell culture.

7. Covalent Modifications of Apo-2 Polypeptides

Covalent modifications of Apo-2 are included within the scope of this invention. One type of covalent modification of the Apo-2 is introduced into the molecule by reacting targeted amino acid residues of the Apo-2 with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of the Apo-2.

Derivatization with bifunctional agents is useful for crosslinking Apo-2 to a water-insoluble support matrix or surface for use in the method for purifying anti-Apo-2 antibodies, and

vice-versa. Derivatization with one or more bifunctional agents will also be useful for crosslinking Apo-2 molecules to generate Apo-2 dimers. Such dimers may increase binding avidity and extend half-life of the molecule *in vivo*. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)-dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

Another type of covalent modification of the Apo-2 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence Apo-2, and/or adding one or more glycosylation sites that are not present in the native sequence Apo-2.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the Apo-2 polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apo-2 (for O-linked glycosylation sites). The Apo-2 amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apo-2 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate moieties on the Apo-2 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-2 polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. For instance, chemical deglycosylation by exposing the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of Apo-2 comprises linking the Apo-2 polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

8. Apo-2 Chimeras

The present invention also provides chimeric molecules comprising Apo-2 fused to another, heterologous polypeptide or amino acid sequence.

In one embodiment, the chimeric molecule comprises a fusion of the Apo-2 with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the Apo-2. The presence of such epitope-tagged forms of the Apo-2 can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apo-2

to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

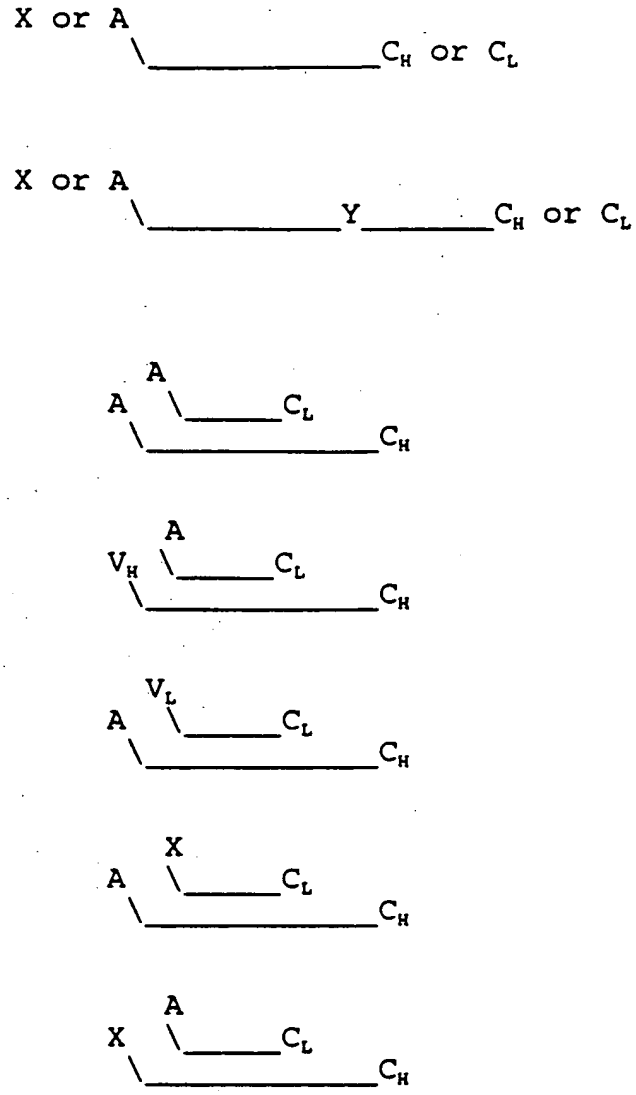
Various tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an α-tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

Generally, epitope-tagged Apo-2 may be constructed and produced according to the methods described above. Epitope-tagged Apo-2 is also described in the Examples below. Apo-2-tag polypeptide fusions are preferably constructed by fusing the cDNA sequence encoding the Apo-2 portion in-frame to the tag polypeptide DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the Apo-2-tag polypeptide chimeras of the present invention, nucleic acid encoding the Apo-2 will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions are also possible. For example, a polyhistidine sequence of about 5 to about 10 histidine residues may be fused at the N- terminus or the C- terminus and used as a purification handle in affinity chromatography.

Epitope-tagged Apo-2 can be purified by affinity chromatography using the anti-tag antibody. The matrix to which

the affinity antibody is attached may include, for instance, agarose, controlled pore glass or poly(styrenedivinyl)benzene. The epitope-tagged Apo-2 can then be eluted from the affinity column using techniques known in the art.

In another embodiment, the chimeric molecule comprises an Apo-2 polypeptide fused to an immunoglobulin sequence. The chimeric molecule may also comprise a particular domain sequence of Apo-2, such as the extracellular domain sequence of native Apo-2 fused to an immunoglobulin sequence. This includes chimeras in monomeric, homo- or heteromultimeric, and particularly homo- or heterodimeric, or -tetrameric forms; optionally, the chimeras may be in dimeric forms or homodimeric heavy chain forms. Generally, these assembled immunoglobulins will have known unit structures as represented by the following diagrams.



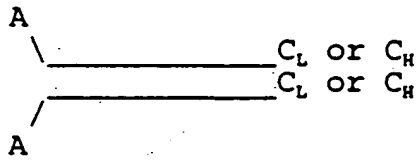
A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in serum. In the case of multimers, each four chain unit may be the same or different.

The following diagrams depict some exemplary monomer, homo- and heterodimer and homo- and heteromultimer structures. These diagrams are merely illustrative, and the chains of the

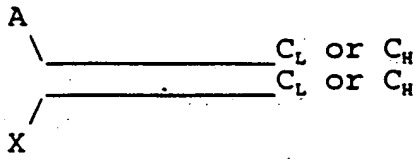
multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins.

monomer:

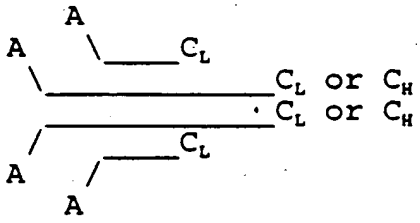
homodimer:



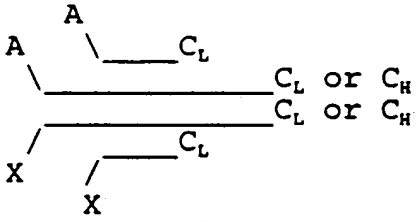
heterodimer:



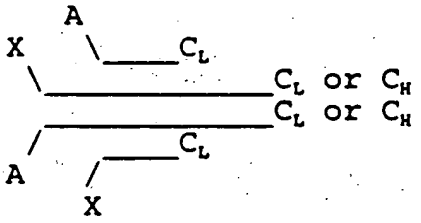
homotetramer:



heterotetramer:



and



In the foregoing diagrams, "A" means an Apo-2 sequence or an Apo-2 sequence fused to a heterologous sequence; X is an additional agent, which may be the same as A or different, a

portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such a pseudomonas exotoxin or ricin, or a sequence functionally binding to another protein, such as other cytokines (i.e., IL-1, interferon-γ) or cell surface molecules (i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma poxviruses), or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; Y is a linker or another receptor sequence; and V_L, V_H, C_L and C_H represent light or heavy chain variable or constant domains of an immunoglobulin. Structures comprising at least one CRD of an Apo-2 sequence as "A" and another cell-surface protein having a repetitive pattern of CRDs (such as TNFR) as "X" are specifically included.

It will be understood that the above diagrams are merely exemplary of the possible structures of the chimeras of the present invention, and do not encompass all possibilities. For example, there might desirably be several different "A"s, "X"s, or "Y"s in any of these constructs. Also, the heavy or light chain constant domains may be originated from the same or different immunoglobulins. All possible permutations of the illustrated and similar structures are all within the scope of the invention herein.

In general, the chimeric molecules can be constructed in a fashion similar to chimeric antibodies in which a variable domain from an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; EP 173,494; Munro, Nature, 312:597 (13 December 1984); Neuberger et al., Nature, 312:604-608 (13 December 1984); Sharon et al., Nature, 309:364-367 (24 May 1984); Morrison et al., Proc. Nat'l. Acad. Sci. USA, 81:6851-6855 (1984); Morrison et al., Science, 229:1202-1207 (1985); Boulianne et al., Nature, 312:643-646 (13 December 1984); Capon et al., Nature, 337:525-531 (1989); Traunecker et al., Nature, 339:68-70 (1989).

Alternatively, the chimeric molecules may be constructed

as follows. The DNA including a region encoding the desired sequence, such as an Apo-2 and/or TNFR sequence, is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point at or near the DNA encoding the N-terminal end of the Apo-2 or TNFR polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for TNFR (where the native signal is employed). This DNA fragment then is readily inserted proximal to DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, the resulting construct tailored by deletional mutagenesis. Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams et al., Biochemistry, 19:2711-2719 (1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al., Proc. Natl. Acad. Sci.. USA, 77:6027-6031 (1980); Rice et al., Proc. Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-288 (1982); and Morrison et al., Ann. Rev. Immunol., 2:239-256 (1984).

Further details of how to prepare such fusions are found in publications concerning the preparation of immunoadhesins. Immunoadhesins in general, and CD4-Ig fusion molecules specifically are disclosed in WO 89/02922, published 6 April 1989). Molecules comprising the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), linked to IgG heavy chain constant region are known in the art and have been found to have a markedly longer half-life and lower clearance than the soluble extracellular portion of CD4 [Capon et al., supra; Byrn et al., Nature, 344:667 (1990)]. The construction of specific chimeric TNFR-IgG molecules is also described in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991); Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P 432)]; and Peppel and Beutler, J. Cell. Biochem. Supplement 15F, 1991, p. 118 (P 439)].

B. Therapeutic and Non-therapeutic Uses for Apo-2

Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using *in vivo* or *ex vivo* gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing the extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF-κB induction by Apo-2L or by another ligand that Apo-2 binds to.

The Apo-2 of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2 may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2 is present in the cell type(s) being evaluated. Apo-2 nucleic acid will also be useful for the preparation of Apo-2 by the recombinant techniques described herein.

The isolated Apo-2 may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of Apo-2 may be prepared. Apo-2 preparations are also useful in generating antibodies, as standards in assays for Apo-2 (*e.g.*, by labeling Apo-2 for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.

Nucleic acids which encode Apo-2 or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal

(e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding Apo-2 or an appropriate sequence thereof (such as Apo-2-IgG) can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding Apo-2. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for Apo-2 transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding Apo-2 introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding Apo-2. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with excessive apoptosis. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition. In another embodiment, transgenic animals that carry a soluble form of Apo-2 such as the Apo-2 ECD or an immunoglobulin chimera of such form could be constructed to test the effect of chronic neutralization of Apo-2L, a ligand of Apo-2.

Alternatively, non-human homologues of Apo-2 can be used to construct an Apo-2 "knock out" animal which has a defective or altered gene encoding Apo-2 as a result of homologous recombination between the endogenous gene encoding Apo-2 and altered genomic DNA encoding Apo-2 introduced into an embryonic cell of the animal. For example, cDNA encoding Apo-2 can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques. A

portion of the genomic DNA encoding Apo-2 can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., Cell, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the Apo-2 polypeptide, including for example, development of tumors.

C. Anti-Apo-2 Antibody Preparation

The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

1. Polyclonal Antibodies

The Apo-2 antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or

adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a Apo-2-IgG fusion protein or chimeric molecule (including an Apo-2 ECD-IgG fusion protein). Cells expressing Apo-2 at their surface may also be employed. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

2. Monoclonal Antibodies

The Apo-2 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, supra. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a Apo-2-IgG fusion protein or chimeric molecule. Cells expressing Apo-2 at their surface may also be employed. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or

lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against Apo-2. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are

known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody

of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain (CH_1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH_1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

3. Humanized Antibodies

The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric

immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567),

wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901 (1987)]. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can

be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/04679 published 3 March 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993)]. Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cote et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cote et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)].

4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have

different specificities [Millstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in

only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [US Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

D. Therapeutic and Non-therapeutic Uses for Apo-2 Antibodies

The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. Alternatively, antagonistic antibodies may be used to block excessive apoptosis (for instance in neurodegenerative disease) or to block potential autoimmune/inflammatory effects of Apo-2 resulting from NF-κB activation.

Apo-2 antibodies may further be used in diagnostic assays for Apo-2, *e.g.*, detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled

with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

Apo-2 antibodies also are useful for the affinity purification of Apo-2 from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2 are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Apo-2, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2 from the antibody.

E. Kits Containing Apo-2 or Apo-2 Antibodies

In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition is Apo-2 or an Apo-2 antibody. The label

on the container indicates that the composition is used for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

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The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Rockville, Maryland.

EXAMPLE 1

Isolation of cDNA clones Encoding Human Apo-2

Expressed sequence tag (EST) DNA databases (LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) were searched and an EST was identified which showed homology to the death domain of the Apo-3 receptor [Marsters et al., Curr. Biol., 6:750 (1996)]. Human pancreas and kidney lgt10 bacteriophage cDNA libraries (both purchased from Clontech) were ligated into pRK5 vectors as follows.

Reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (3 ml); pRK5, Xho1, Not1 digested vector, 0.5 mg, 1 ml); cDNA (5 ml) and distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) were added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was removed, collected and diluted into 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, and the pellet resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The DNA pellet was then dried in a speedvac and eluted into distilled water (3 ml) for use in the subsequent procedure.

The ligated cDNA/pRK5 vector DNA prepared previously was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then electroporated as per the manufacturers recommendation. Subsequently SOC media (1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C) to allow the colonies to grow. Positive colonies were then scraped off and the DNA isolated from the bacterial pellet using standard CsCl-gradient protocols.

An enriched 5'-cDNA library was then constructed to obtain a bias of cDNA fragments which preferentially represents the 5' ends of cDNA's contained within the library. 10 mg of the pooled isolated full-length library plasmid DNA (41 ml) was combined with Not 1 restriction buffer (New England Biolabs, 5 ml) and Not 1 (New England Biolabs, 4 ml) and incubated at 37°C for one hour. The reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml), the aqueous phase removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was then removed, the pellet dried in a speedvac and resuspended in

distilled water (10 ml).

The following reagents were brought together and incubated at 37°C for 2 hours: distilled water (3 ml); linearized DNA library (1 mg, 1 ml); Ribonucleotide mix (Invitrogen, 10 ml); transcription buffer (Invitrogen, 2 ml) and Sp6 enzyme mix. The reaction was then extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml) and the aqueous phase was removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml) and centrifuged for 20 minutes at 14,000 x g. The pellet was then decanted and resuspended in 70% ethanol (0.5 ml), centrifuged again for 2 minutes at 14,000 x g, decanted, dried in a speedvac and resuspended into distilled water (10 ml).

The following reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (Life Tech., 3 ml); pRK5 Cla-Sal digested vector, 0.5 mg, 1 ml); cDNA (5 ml); distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) was added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 100 ml). The aqueous phase was removed, collected and diluted by 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml) and centrifuged for 20 minutes at 14,000 x g. The DNA pellet was decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was removed and the residue pellet was dried in a speedvac and resuspended in distilled water (3ml). The ligated cDNA/pSST-amy.1 vector DNA was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then electroporated as recommended by the manufacturer. Subsequently, SOC media (Life Tech., 1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C). Positive colonies were scraped off the plates and the DNA was isolated from the bacterial pellet using standard protocols, e.g. CsCl-gradient.

The cDNA libraries were screened by hybridization with a

synthetic oligonucleotide probe:
GGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGGCA
GCGGG (SEQ ID NO:3) based on the EST.

Three cDNA clones were sequenced in entirety. The overlapping coding regions of the cDNAs were identical except for codon 410 (using the numbering system for Fig. 1); this position encoded a leucine residue (TTG) in both pancreatic cDNAs, and a methionine residue (ATG) in the kidney cDNA, possibly due to polymorphism.

The entire nucleotide sequence of Apo-2 is shown in Figure 1 (SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-2 deposited as ATCC _____, as indicated below) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 140-142 [Kozak et al., supra] and ending at the stop codon found at nucleotide positions 1373-1375 (Fig. 1; SEQ ID NO:2). The predicted polypeptide precursor is 411 amino acids long, a type I transmembrane protein, and has a calculated molecular weight of approximately 45 kDa. Hydropathy analysis (not shown) suggested the presence of a signal sequence (residues 1-53), followed by an extracellular domain (residues 54-182), a transmembrane domain (residues 183-208), and an intracellular domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid sequence analysis of Apo-2-IgG expressed in 293 cells showed that the mature polypeptide starts at amino acid residue 54, indicating that the actual signal sequence comprises residues 1-53.

TNF receptor family proteins are typically characterized by the presence of multiple (usually four) cysteine-rich domains in their extracellular regions -- each cysteine-rich domain being approximately 45 amino acids long and containing approximately 6, regularly spaced, cysteine residues. Based on the crystal structure of the type 1 TNF receptor, the cysteines in each domain typically form three disulfide bonds in which usually cysteines 1 and 2, 3 and 5, and 4 and 6 are paired together. Like DR4, Apo-2 contains two extracellular cysteine-rich pseudorepeats (Fig. 2A), whereas other identified mammalian TNFR family members contain

three or more such domains [Smith et al., Cell, 76:959 (1994)].

The cytoplasmic region of Apo-2 contains a death domain (amino acid residues 324-391 shown in Fig. 1; see also Fig. 2A) which shows significantly more amino acid sequence identity to the death domain of DR4 (64%) than to the death domain of TNFR1 (30%); CD95 (19%); or Apo-3/DR3 (29%) (Fig. 2B). Four out of six death domain amino acids that are required for signaling by TNFR1 [Tartaglia et al., supra] are conserved in Apo-2 while the other two residues are semi-conserved (see Fig. 2B).

Based on an alignment analysis (using the ALIGN™ computer program) of the full-length sequence, Apo-2 shows more sequence identity to DR4 (55%) than to other apoptosis-linked receptors, such as TNFR1 (19%); CD95 (17%); or Apo-3 (also referred to as DR3, WSL-1 or TRAMP) (29%).

EXAMPLE 2

A. Expression of Apo-2 ECD

A soluble extracellular domain (ECD) fusion construct was prepared. An Apo-2 ECD (amino acid residues 1-184 shown in Figure 1) was obtained by PCR and fused to a C-terminal Flag epitope tag (Sigma). (The Apo-2 ECD construct included residues 183 and 184 shown in Figure 1 to provide flexibility at the junction, even though residues 183 and 184 are predicted to be in the transmembrane region). The Flag epitope-tagged molecule was then inserted into pRK5, and expressed by transient transfection into human 293 cells (ATCC CRL 1573).

After a 48 hour incubation, the cell supernatants were collected and either used directly for co-precipitation studies (see Example 3) or subjected to purification of the Apo-2 ECD-Flag by affinity chromatography on anti-Flag agarose beads, according to manufacturer's instructions (Sigma).

B. Expression of Apo-2 ECD as an Immunoadhesin

A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the

hinge and Fc region of human immunoglobulin G_1 heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra.

EXAMPLE 3

Immunoprecipitation Assay Showing Binding Interaction Between Apo-2 and Apo-2 Ligand

To determine whether Apo-2 and Apo-2L interact or associate with each other, supernatants from mock-transfected 293 cells or from 293 cells transfected with Apo-2 ECD-Flag (described in Example 2 above) (5 ml) were incubated with 5 μg poly-histidine-tagged soluble Apo-2L [Pitti et al., supra] for 30 minutes at room temperature and then analyzed for complex formation by a co-precipitation assay.

The samples were subjected to immunoprecipitation using 25 μl anti-Flag conjugated agarose beads (Sigma) or Nickel-conjugated agarose beads (Qiagen). After a 1.5 hour incubation at 4° C, the beads were spun down and washed four times in phosphate buffered saline (PBS). By using anti-Flag agarose, the Apo-2L was precipitated through the Flag-tagged Apo-2 ECD; by using Nickel-agarose, the Apo-2 ECD was precipitated through the His-tagged Apo-2L. The precipitated proteins were released by boiling the beads for 5 minutes in SDS-PAGE buffer, resolved by electrophoresis on 12% polyacrylamide gels, and then detected by immunoblot with anti-Apo-2L or anti-Flag antibody (2 μg/ml) as described in Marsters et al., J. Biol. Chem., (1997).

The results, shown in Figure 3, indicate that the Apo-2 ECD and Apo-2L can associate with each other.

The binding interaction was further analyzed by purifying Apo-2 ECD from the transfected 293 cell supernatants with anti-Flag beads (see Example 2) and then analyzing the samples on a BIACORE™ instrument. The BIACORE™ analysis indicated a dissociation

constant (K_d) of about 1 nM. BIACORE™ analysis also showed that the Apo-2 ECD is not capable of binding other apoptosis-inducing TNF family members, namely, TNF-alpha (Genentech, Inc., Pennica et al., Nature, 312:712 (1984), lymphotoxin-alpha (Genentech, Inc.), or Fas/Apo-1 ligand (Alexis Biochemicals). The data thus shows that Apo-2 is a specific receptor for Apo-2L.

EXAMPLE 4

Induction of Apoptosis by Apo-2

Because death domains can function as oligomerization interfaces, over-expression of receptors that contain death domains may lead to activation of signaling in the absence of ligand [Frazer et al., supra, Nagata et al., supra]. To determine whether Apo-2 was capable of inducing cell death, human 293 cells or HeLa cells (ATCC CCL 2.2) were transiently transfected by calcium phosphate precipitation (293 cells) or electroporation (HeLa cells) with a pRK5 vector or pRK5-based plasmids encoding Apo-2 and/or CrmA. When applicable, the total amount of plasmid DNA was adjusted by adding vector DNA. Apoptosis was assessed 24 hours after transfection by morphology (Fig. 4A); DNA fragmentation (Fig. 4B); or by FACS analysis of phosphatydilserine exposure (Fig. 4C) as described in Marsters et al., Curr. Biol., 6:1669 (1996). As shown in Figs. 4A and 4B, the Apo-2 transfected 293 cells underwent marked apoptosis.

For samples assayed by FACS, the HeLa cells were co-transfected with pRK5-CD4 as a marker for transfection and apoptosis was determined in CD4-expressing cells; FADD was co-transfected with the Apo-2 plasmid; the data are means ± SEM of at least three experiments, as described in Marsters et al., Curr. Biol., 6:1669 (1996). The caspase inhibitors, DEVD-fmk (Enzyme Systems) or z-VAD-fmk (Research Biochemicals Intl.) were added at 200 μM at the time of transfection. As shown in Fig. 4C, the caspase inhibitors CrmA, DEVD-fmk, and z-VAD-fmk blocked apoptosis induction by Apo-2, indicating the involvement of Ced-3-like proteases in this response.

FADD is an adaptor protein that mediates apoptosis activation by CD95, TNFR1, and Apo-3/DR3 [Nagata et al., supra], but does not appear necessary for apoptosis induction by Apo-2L [Marsters et al., supra] or by DR4 [Pan et al., supra]. A dominant-negative mutant form of FADD, which blocks apoptosis induction by CD95, TNFR1, or Apo-3/DR3 [Frazer et al., supra; Nagata et al., supra; Chinnayian et al., supra] did not inhibit apoptosis induction by Apo-2 when co-transfected into HeLa cells with Apo-2 (Fig. 4C). These results suggest that Apo-2 signals apoptosis independently of FADD. Consistent with this conclusion, a glutathione-S-transferase fusion protein containing the Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and translated FADD (data not shown).

EXAMPLE 5

Inhibition of Apo-2L Activity by Soluble Apo-2 ECD

Soluble Apo-2L (0.5 μg/ml, prepared as described in Pitti et al., supra) was pre-incubated for 1 hour at room temperature with PBS buffer or affinity-purified Apo-2 ECD (5 μg/ml) together with anti-Flag antibody (Sigma) (1 μg/ml) and added to HeLa cells. After a 5 hour incubation, the cells were analyzed for apoptosis by FACS (as above) (Fig. 4D).

Apo-2L induced marked apoptosis in HeLa cells, and the soluble Apo-2 ECD was capable of blocking Apo-2L action (Fig. 4D), confirming a specific interaction between Apo-2L and Apo-2. Similar results were obtained with the Apo-2 ECD immunoadhesin (Fig. 4D). Dose-response analysis showed half-maximal inhibition at approximately 0.3 nM Apo-2 immunoadhesin (Fig. 4E).

EXAMPLE 6

Activation of NF-κB by Apo-2

An assay was conducted to determine whether Apo-2 activates NF-κB.

HeLa cells were transfected with pRK5 expression plasmids encoding full-length native sequence Apo-2, DR4 or Apo-3 and

harvested 24 hours after transfection. Nuclear extracts were prepared and 1 μg of nuclear protein was reacted with a ^{32}P-labelled NF-κB-specific synthetic oligonucleotide ATCAGGGACTTTCCGCTGGGGACTTTCCG (SEQ ID NO:4) [see, also, MacKay et al., J. Immunol., 153:5274-5284 (1994)], alone or together with a 50-fold excess of unlabelled probe, or with an irrelevant ^{32}P-labelled synthetic oligonucleotide AGGATGGGAAGTGTGTGATATATCCTTGAT (SEQ ID NO:5). In some samples, antibody to p65/RelA subunits of NF-κB (1 μg/ml; Santa Cruz Biotechnology) was added. DNA binding was analyzed by an electrophoretic mobility shift assay as described by Hsu et al., supra; Marsters et al., supra, and MacKay et al., supra.

The results are shown in Fig. 5. As shown in Fig. 5A, upon transfection into HeLa cells, both Apo-2 and DR4 induced significant NF-κB activation as measured by the electrophoretic mobility shift assay; the level of activation was comparable to activation observed for Apo-3/DR3. Antibody to the p65/RelA subunit of NF-κB inhibited the mobility of the NF-κB probe, implicating p65 in the response to all 3 receptors.

An assay was also conducted to determine if Apo-2L itself can regulate NF-κB activity. HeLa cells or MCF7 cells (human breast adenocarcinoma cell line, ATCC HTB 22) were treated with PBS buffer, soluble Apo-2L (Pitti et al., supra) or TNF-alpha (Genentech, Inc., see Pennica et al., Nature, 312:721 (1984)) (1 μg/ml) and assayed for NF-κB activity as above. The results are shown in Fig. 5B. The Apo-2L induced a significant NF-κB activation in the treated HeLa cells but not in the treated MCF7 cells; the TNF-alpha induced a more pronounced activation in both cell lines. Several studies have disclosed that NF-κB activation by TNF can protect cells against TNF-induced apoptosis [Nagata, supra].

The effects of a NF-κB inhibitor, ALLN (N-acetyl-Leu-Leu-norleucinal) and a transcription inhibitor, cyclohexamide, were also tested. The HeLa cells (plated in 6-well dishes) were preincubated with PBS buffer, ALLN (Calbiochem) (40 μg/ml) or

cyclohexamide (Sigma) (50 μg/ml) for 1 hour before addition of Apo-2L (1 μg/ml). After a 5 hour incubation, apoptosis was analyzed by FACS (see Fig. 5C).

The results are shown in Fig. 5C. Both ALLN and cyclohexamide increased the level of Apo-2L-induced apoptosis in the HeLa cells. The data indicates that Apo-2L can induce protective NF-κB-dependent genes. The data also indicates that Apo-2L is capable of activating NF-κB in certain cell lines and that both Apo-2 and DR4 may mediate that function.

EXAMPLE 7

Northern Blot Analysis

Expression of Apo-2 mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a 4.6 kilobase ^{32}P-labelled DNA probe based on the full length Apo-2 cDNA; the probe was generated by digesting the pRK5-Apo-2 plasmid with EcoRI. Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure.

As shown in Fig. 6, a predominant mRNA transcript of approximately 4.6kb was detected in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary and peripheral blood leukocytes (PBL), while no mRNA expression was detected in fetal and adult brain. Intermediate levels of expression were seen in adult colon, small intestine, testis, prostate, thymus, pancreas, lidney, skeletal muscle, placenta, and heart. Several adult tissues that express Apo-2, e.g., PBL, ovary, and spleen, have been shown previously to express DR4 [Pan et al., supra], however, the relative levels of expression of each receptor mRNA appear to be different.

EXAMPLE 8

Chromosomal Localization of the Apo-2 gene

Chromosomal localization of the human Apo-2 gene was examined by radiation hybrid (RH) panel analysis. RH mapping was performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-2 cDNA [Gelb et al., Hum. Genet., 98:141 (1996)]. Analysis of the PCR data using the Stanford Human Genome Center Database indicates that Apo-2 is linked to the marker D8S481, with an LOD of 11.05; D8S481 is linked in turn to D8S2055, which maps to human chromosome 8p21. A similar analysis of DR4 showed that DR4 is linked to the marker D8S2127 (with an LOD of 13.00), which maps also to human chromosome 8p21.

To Applicants' present knowledge, to date, no other member of the TNFR gene family has been located to chromosome 8.

* * * * *

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

Material	ATCC Dep. No.	Deposit Date
pRK5-Apo-2	_____	_____

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the

invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Ashkenazi, Avi J.

(ii) TITLE OF INVENTION: Apo-2 RECEPTOR

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 460 Point San Bruno Blvd
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 15-May-1997
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Marschang, Diane L.
(B) REGISTRATION NUMBER: 35,600
(C) REFERENCE/DOCKET NUMBER: P1101

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 415/225-5416
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 411 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro
20 25 30

Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val
35 40 45

Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp
50 55 60

Leu Ala Pro Gln Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser
65 70 75

Pro Ser Glu Gly Leu Cys Pro Pro Gly His His Ile Ser Glu Asp
80 85 90

Gly Arg Asp Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr
95 100 105

His Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys Asp

110 115 120

Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr Arg Asn Thr
125 130 135

Val Cys Gln Cys Glu Glu Gly Thr Phe Arg Glu Glu Asp Ser Pro
140 145 150

Glu Met Cys Arg Lys Cys Arg Thr Gly Cys Pro Arg Gly Met Val
155 160 165

Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile Glu Cys Val His
170 175 180

Lys Glu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val
185 190 195

Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys
200 205 210

Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp
215 220 225

Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp
230 235 240

Asn Val Leu Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val
245 250 255

Pro Glu Gln Glu Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly
260 265 270

Val Asn Met Leu Ser Pro Gly Glu Ser Glu His Leu Leu Glu Pro
275 280 285

Ala Glu Ala Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala
290 295 300

Asn Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp
305 310 315

Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg
320 325 330

Lys Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu
335 340 345

Ala Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp
350 355 360

Val Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp
365 370 375

Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu
380 385 390

Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn
395 400 405

Ala Asp Ser Ala Xaa Ser
410 411

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1799 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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                                           Met Glu
                                             1

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Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg
          5                  10                  15

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Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala
                20                  25

AGG CCT GGG CTC CGG GTC CCC AAG ACC CTT GTG CTC GTT 262
Arg Pro Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val
     30                  35                  40

GTC GCC GCG GTC CTG CTG TTG GTC TCA GCT GAG TCT GCT 301
Val Ala Ala Val Leu Leu Leu Val Ser Ala Glu Ser Ala
               45                  50

CTG ATC ACC CAA CAA GAC CTA GCT CCC CAG CAG AGA GCG 340
Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln Gln Arg Ala
 55                  60                  65

GCC CCA CAA CAA AAG AGG TCC AGC CCC TCA GAG GGA TTG 379
Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu
          70                  75                  80
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TGT CCA CCT GGA CAC CAT ATC TCA GAA GAC GGT AGA GAT 418
Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp
                  85                  90

TGC ATC TCC TGC AAA TAT GGA CAG GAC TAT AGC ACT CAC 457
Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His
     95                 100                 105

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Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys
             110                 115

GAT TCA GGT GAA GTG GAG CTA AGT CCC TGC ACC ACG ACC 535
Asp Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr
120                 125                 130

AGA AAC ACA GTG TGT CAG TGC GAA GAA GGC ACC TTC CGG 574
Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe Arg
        135                 140                 145

GAA GAA GAT TCT CCT GAG ATG TGC CGG AAG TGC CGC ACA 613
Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr
                  150                 155

GGG TGT CCC AGA GGG ATG GTC AAG GTC GGT GAT TGT ACA 652
Gly Cys Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr
    160                 165                 170

CCC TGG AGT GAC ATC GAA TGT GTC CAC AAA GAA TCA GGC 691
Pro Trp Ser Asp Ile Glu Cys Val His Lys Glu Ser Gly
             175                 180

ATC ATC ATA GGA GTC ACA GTT GCA GCC GTA GTC TTG ATT 730
Ile Ile Ile Gly Val Thr Val Ala Ala Val Val Leu Ile
185                 190                 195
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GTC CTT CCT TAC CTG AAA GGC ATC TGC TCA GGT GGT GGT 808
Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly
                215                 220

GGG GAC CCT GAG CGT GTG GAC AGA AGC TCA CAA CGA CCT 847
Gly Asp Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro
    225                 230                 235

GGG GCT GAG GAC AAT GTC CTC AAT GAG ATC GTG AGT ATC 886
Gly Ala Glu Asp Asn Val Leu Asn Glu Ile Val Ser Ile
            240                 245

TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA ATG GAA GTC 925
Leu Gln Pro Thr Gln Val Pro Glu Gln Glu Met Glu Val
250                 255                 260

CAG GAG CCA GCA GAG CCA ACA GGT GTC AAC ATG TTG TCC 964
Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser
        265                 270                 275

CCC GGG GAG TCA GAG CAT CTG CTG GAA CCG GCA GAA GCT 1003
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                280                 285

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Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala Asn
    290                 295                 300

GAA GGT GAT CCC ACT GAG ACT CTG AGA CAG TGC TTC GAT 1081
Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp
            305                 310
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315                 320                 325

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Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu Ile Lys
        330                 335                 340

GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG 1198
Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu
                345                 350

TAC ACG ATG CTG ATA AAG TGG GTC AAC AAA ACC GGG CGA 1237
Tyr Thr Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg
    355                 360                 365

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380                 385                 390

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Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn
        395                 400                 405

GCA GAC TCT GCC WTG TCC TAAGTGTG ATTCTCTTCA GGAAGTGAGA 1400
Ala Asp Ser Ala Xaa Ser
                410 411

CCTTCCCTGG TTTACCTTTT TTCTGGAAAA AGCCCAACTG GACTCCAGTC 1450

AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC 1500
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CCATCCAACA TCACCCAGTG GATGGAACAT CCTGTAACTT TTCACTGCAC 1550

TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT 1600

GTCTGGATCA TTCCGTTTGT GCGTACTTTG AGATTTGGTT TGGGATGTCA 1650

TTGTTTTCAC AGCACTTTTT TATCCTAATG TAAATGCTTT ATTTATTTAT 1700

TTGGGCTACA TTGTAAGATC CATCTACAAA AAAAAAAAAA AAAAAAAAAG 1750

GGCGGCCGCG ACTCTAGAGT CGACCTGCAG AAGCTTGGCC GCCATGGCC 1799

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGAGCCGCT CATGAGGAAG TTGGGCCTCA TGGACAATGA GATAAAGGTG 50

GCTAAAGCTG AGGCAGCGGG 70

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATCAGGGACT TTCCGCTGGG GACTTTCCG 29

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGGATGGGAA GTGTGTGATA TATCCTTGAT 30

WHAT IS CLAIMED IS:

1. A method of modulating apoptosis in mammalian cells comprising exposing mammalian cells to Apo-2 polypeptide, said Apo-2 polypeptide having at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising amino acid residues 1 to 411 of SEQ ID NO:1.

2. A method of modulating apoptosis in mammalian cells comprising exposing mammalian cells to an extracellular domain sequence of Apo-2 polypeptide comprising amino acid residues 54 to 182 of SEQ ID NO:1.

Abstract of the Disclosure

Novel polypeptides, designated Apo-2, which are capable of modulating apoptosis are provided. Compositions including Apo-2 chimeras, nucleic acid encoding Apo-2, and antibodies to Apo-2 are also provided.

Fig. 1

```
   1 CCCACGCGTC CGCATAAATC AGCACGCGGC CGGAGAACCC CGCAATCTCT GCGCCCACAA AATACACCGA CGATGCCCGA TCTACTTTAA GGGCTGAAAC
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 101 CCACGGGCCT GAGAGACTAT AAGAGCGTTC CCTACCGCCA TGGAACAACG GGGACAGAAC GCCCCGGCCG CTTCGGGGGC CCGGAAAAGG CACGGCCCAG
     GGTGCCCGGA CTCTCTGATA TTCTCGCAAG GGATGGCGGT ACCTTGTTGC CCCTGTCTTG CGGGGCCGGC GAAGCCCCCG GGCCTTTTCC GTGCCGGGTC
   1                                                M etGluGlnAr gGlyGlnAsn AlaProAlaA laSerGlyAl aArgLysArg HisGlyProGly

 201 GACCCAGGGA GGCGCGGGGA GCCAGGCCTG GGCTCCGGGT CCCCAAGACC CTTGTGCTCG TTGTCGCCGC GGTCCTGCTG TTGGTCTCAG CTGAGTCTGC
     CTGGGTCCCT CCGCGCCCCT CGGTCCGGAC CCGAGGCCCA GGGGTTCTGG GAACACGAGC AACAGCGGCG CCAGGACGAC AACCAGAGTC GACTCAGACG
  22   ProArgGl uAlaArgGly AlaArgProG lyLeuArgVa lProLysThr LeuValLeuV alValAlaAl aValLeuLeu LeuValSerA laGluSerAla

 301 TCTGATCACC CAACAAGACC TAGCTCCCCA GCAGAGAGCG GCCCCACAAC AAAAGAGGTC CAGCCCCTCA GAGGGATTGT GTCCACCTGG ACACCATATC
     AGACTAGTGG GTTGTTCTGG ATCGAGGGGT CGTCTCTCGC CGGGGTGTTG TTTTCTCCAG GTCGGGGAGT CTCCCTAACA CAGGTGGACC TGTGGTATAG
  55  LeuIleThr GlnGlnAspL euAlaProGl nGlnArgAla AlaProGlnG lnLysArgSe rSerProSer GluGlyLeuC ysProProGl yHisHisIle

 401 TCAGAAGACG GTAGAGATTG CATCTCCTGC AAATATGGAC AGGACTATAG CACTCACTGG AATGACCTCC TTTTCTGCTT GCGCTGCACC AGGTGTGATT
     AGTCTTCTGC CATCTCTAAC GTAGAGGACG TTTATACCTG TCCTGATATC GTGAGTGACC TTACTGGAGG AAAAGACGAA CGCGACGTGG TCCACACTAA
  88 SerGluAspG lyArgAspCy sIleSerCys LysTyrGlyG lnAspTyrSe rThrHisTrp AsnAspLeuL euPheCysLe uArgCysThr ArgCysAspSer

 501 CAGGTGAAGT GGAGCTAAGT CCCTGCACCA CGACCAGAAA CACAGTGTGT CAGTGCGAAG AAGGCACCTT CCGGGAAGAA GATTCTCCTG AGATGTGCCG
     GTCCACTTCA CCTCGATTCA GGGACGTGGT GCTGGTCTTT GTGTCACACA GTCACGCTTC TTCCGTGGAA GGCCCTTCTT CTAAGAGGAC TCTACACGGC
 122   GlyGluVa lGluLeuSer ProCysThrT hrThrArgAs nThrValCys GlnCysGluG luGlyThrPh eArgGluGlu AspSerProG luMetCysArg

 601 GAAGTGCCGC ACAGGGTGTC CCAGAGGGAT GGTCAAGGTC GGTGATTGTA CACCCTGGAG TGACATCGAA TGTGTCCACA AAGAATCAGG CATCATCATA
     CTTCACGGCG TGTCCCACAG GGTCTCCCTA CCAGTTCCAG CCACTAACAT GTGGGACCTC ACTGTAGCTT ACACAGGTGT TTCTTAGTCC GTAGTAGTAT
 155  LysCysArg ThrGlyCysP roArgGlyMe tValLysVal GlyAspCysT hrProTrpSe rAspIleGlu CysValHisL ysGluSerGl yIleIleIle

 701 GGAGTCACAG TTGCAGCCGT AGTCTTGATT GTGGCTGTGT TTGTTTGCAA GTCTTTACTG TGGAAGAAAG TCCTTCCTTA CCTGAAAGGC ATCTGCTCAG
     CCTCAGTGTC AACGTCGGCA TCAGAACTAA CACCGACACA AACAAACGTT CAGAAATGAC ACCTTCTTTC AGGAAGGAAT GGACTTTCCG TAGACGAGTC
 188 GlyValThrV alAlaAlaVa lValLeuIle ValAlaValP heValCysLy sSerLeuLeu TrpLysLysV alLeuProTy rLeuLysGly IleCysSerGly

 801 GTGGTGGTGG GGACCCTGAG CGTGTGGACA GAAGCTCACA ACGACCTGGG GCTGAGGACA ATGTCCTCAA TGAGATCGTG AGTATCTTGC AGCCCACCCA
     CACCACCACC CCTGGGACTC GCACACCTGT CTTCGAGTGT TGCTGGACCC CGACTCCTGT TACAGGAGTT ACTCTAGCAC TCATAGAACG TCGGGTGGGT
 222   GlyGlyGl yAspProGlu ArgValAspA rgSerSerGl nArgProGly AlaGluAspA snValLeuAs nGluIleVal SerIleLeuG lnProThrGln

 901 GGTCCCTGAG CAGGAAATGG AAGTCCAGGA GCCAGCAGAG CCAACAGGTG TCAACATGTT GTCCCCCGGG GAGTCAGAGC ATCTGCTGGA ACCGGCAGAA
     CCAGGGACTC GTCCTTTACC TTCAGGTCCT CGGTCGTCTC GGTTGTCCAC AGTTGTACAA CAGGGGGCCC CTCAGTCTCG TAGACGACCT TGGCCGTCTT
 255  ValProGlu GlnGluMetG luValGlnGl uProAlaGlu ProThrGlyV alAsnMetLe uSerProGly GluSerGluH isLeuLeuGl uProAlaGlu

1001 GCTGAAAGGT CTCAGAGGAG GAGGCTGCTG GTTCCAGCAA ATGAAGGTGA TCCCACTGAG ACTCTGAGAC AGTGCTTCGA TGACTTTGCA GACTTGGTGC
     CGACTTTCCA GAGTCTCCTC CTCCGACGAC CAAGGTCGTT TACTTCCACT AGGGTGACTC TGAGACTCTG TCACGAAGCT ACTGAAACGT CTGAACCACG
 288 AlaGluArgS erGlnArgAr gArgLeuLeu ValProAlaA snGluGlyAs pProThrGlu ThrLeuArgG lnCysPheAs pAspPheAla AspLeuValPro
```

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1101 CCTTTGACTC CTGGGAGCCG CTCATGAGGA AGTTGGGCCT CATGGACAAT GAGATAAAGG TGGCTAAAGC TGAGGCAGCG GGCCACAGGG ACACCTTGTA
     GGAAACTGAG GACCCTCGGC GAGTACTCCT TCAACCCGGA GTACCTGTTA CTCTATTTCC ACCGATTTCG ACTCCGTCGC CCGGTGTCCC TGTGGAACAT
 322   PheAspSe rTrpGluPro LeuMetArgL ysLeuGlyLe uMetAspAsn GluIleLysV alAlaLysAl aGluAlaAla GlyHisArgA spThrLeuTyr

1201 CACGATGCTG ATAAAGTGGG TCAACAAAAC CGGGCGAGAT GCCTCTGTCC ACACCCTGCT GGATGCCTTG GAGACGCTGG GAGAGAGACT TGCCAAGCAG
     GTGCTACGAC TATTTCACCC AGTTGTTTTG GCCCGCTCTA CGGAGACAGG TGTGGGACGA CCTACGGAAC CTCTGCGACC CTCTCTCTGA ACGGTTCGTC
 355  ThrMetLeu IleLysTrpV alAsnLysTh rGlyArgAsp AlaSerValH isThrLeuLe uAspAlaLeu GluThrLeuG lyGluArgLe uAlaLysGln

1301 AAGATTGAGG ACCACTTGTT GAGCTCTGGA AAGTTCATGT ATCTAGAAGG TAATGCAGAC TCTGCCWTGT CCTAAGTGTG ATTCTCTTCA GGAAGTGAGA
     TTCTAACTCC TGGTGAACAA CTCGAGACCT TTCAAGTACA TAGATCTTCC ATTACGTCTG AGACGGAACA GGATTCACAC TAAGAGAAGT CCTTCACTCT
 388 LysIleGluA spHisLeuLe uSerSerGly LysPheMetT yrLeuGluGl yAsnAlaAsp SerAlaXqqS erOC*

1401 CCTTCCCTGG TTTACCTTTT TTCTGGAAAA AGCCCAACTG GACTCCAGTC AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC
     GGAAGGGACC AAATGGAAAA AAGACCTTTT TCGGGTTGAC CTGAGGTCAG TCATCCTTTC ACGGTGTTAA CAGTGTACTG GCCATGACCT TCTTTGAGAG

1501 CCATCCAACA TCACCCAGTG GATGGAACAT CCTGTAACTT TTCACTGCAC TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT
     GGTAGGTTGT AGTGGGTCAC CTACCTTGTA GGACATTGAA AAGTGACGTG AACCGTAATA AAAATATTCG ACTTACACTA TTATTCCTGT GATACCTTTA

1601 GTCTGGATCA TTCCGTTTGT GCGTACTTTG AGATTTGGTT TGGGATGTCA TTGTTTTCAC AGCACTTTTT TATCCTAATG TAAATGCTTT ATTTATTTAT
     CAGACCTAGT AAGGCAAACA CGCATGAAAC TCTAAACCAA ACCCTACAGT AACAAAAGTG TCGTGAAAAA ATAGGATTAC ATTTACGAAA TAAATAAATA

1701 TTGGGCTACA TTGTAAGATC CATCTACAAA AAAAAAAAAA AAAAAAAAAG GGCGGCCGCG ACTCTAGAGT CGACCTGCAG AAGCTTGGCC GCCATGGCC
     AACCCGATGT AACATTCTAG GTAGATGTTT TTTTTTTTTT TTTTTTTTTC CCGCCGGCGC TGAGATCTCA GCTGGACGTC TTCGAACCGG CGGTACCGG
```

Fig. 1 (cont.)

FIG. 2A

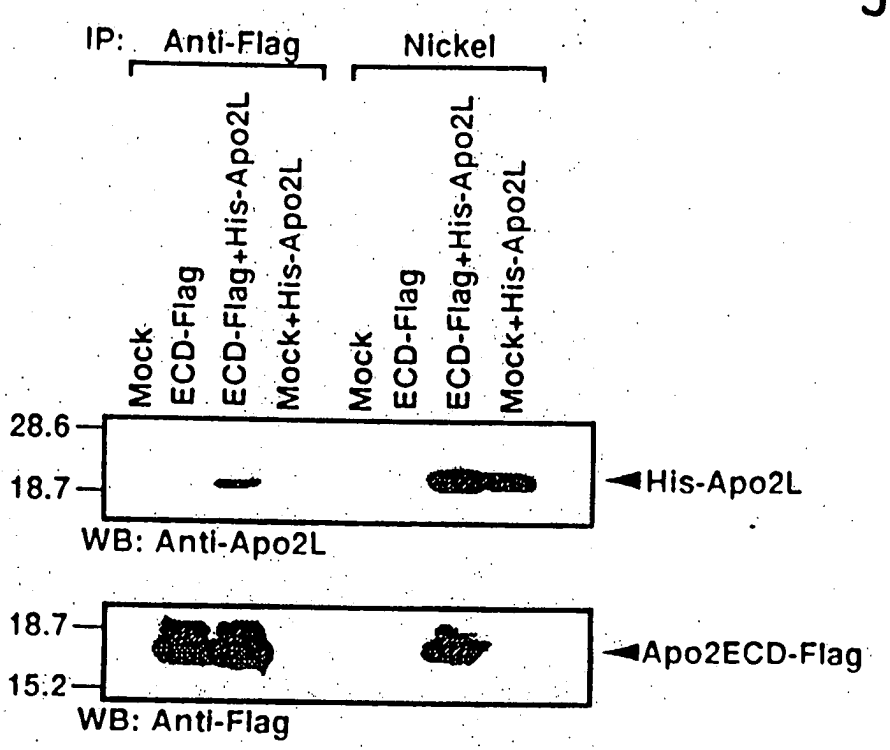
```
  1 MEQRGQNAPAASGARKRHGPGPREARGARPGLRVPKTLVLVVAAVLLLVSAESALITQQD
 61 LAPQQRAAPQQKRSSPSEGLCPPGHHISEDGRDCISCKYGQDYSTHWNDLLFCLRCTRCD
121 SGEVELSPCTTTRNTVCQCEEGTFREEDSPEMCRKCRTGCPRGMVKVGDCTPWSDIECVH
181 KESGIIIGVTVAAVVLIVAVFVCKSLLWKKVLPYLKGICSGGGGDPERVDRSSQRPGAED
241 NVLNEIVSILQPTQVPEQEMEVQEPAEPTGVNMLSPGESEHLLEPAEAERSQRRRLLVPA
301 NEGDPTETLRQCFDDFADLVPFDSWEPLMRKLGLMDNEIKVAKAEAAGHRDTLYTMLIKW
361 VNKTGRDASVHTLLDALETLGERLAKQKIEDHLLSSGKFMYLEGNADSALS
```

FIG. 2B

```
Apo2       FADLVPFDSWEPLMRKLGLMDNEIKVAKAEAA--GHRDTL
DR4        FANIVPFDSWDQLMRQLDLTKNEIDVVRAGTA--GPGDAL
Apo3/DR3   VMDAVPARRWKEFVRTLGLREAEIEAVEVEIGR--FRDQQ
TNFR1      VVENVPPLRWKEFVRRLGLSDHEIDRLELQNGR-CLREAQ
Fas/Apo1   IAGVMTLSQVKGFVRKNGVNEAKIDEIKNDNVQDTAEQKV

Apo2       YTMLIKWVNKTGRD-ASVHTLLDALETLGEELAKQKIED
DR4        YAMLMKWVNKTGRN-ASIHTLLDALERMEERHAKEKIQD
Apo3/DR3   YEMLKRWRQQQP---AGLGAVYAALERMGLDGCVEDLRS
TNFR1      YSMLATWRRRTPRREATLELLGRVLRDMDLLGCLEDIEE
Fas/Apo1   -QLLRNWHQLHGKKEAY-DTLIKDLKKANLCTLAEKIQT
```

Fig. 3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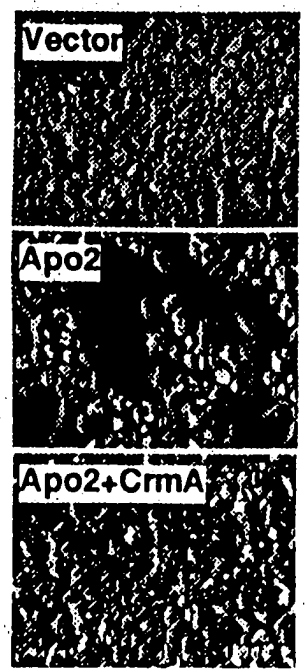
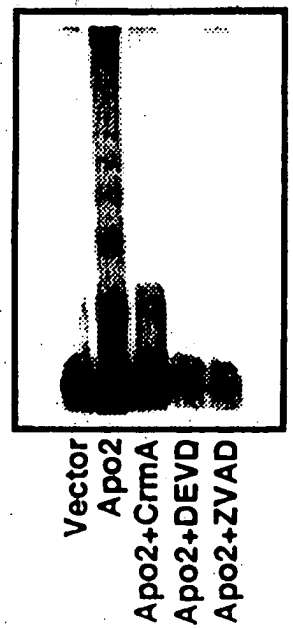
Nickel
Mock
ECD-Flag
ECD-Flag+His-Apo2L
Mock+His-Apo2L
Mock
ECD-Flag
ECD-Flag+His-Apo2L
Mock+His-Apo2L
28.6
18.7
His-Apo2L
WB: Anti-Apo2L
18.7
15.2
Apo2ECD-Flag
WB: Anti-Flag

Fig. 4

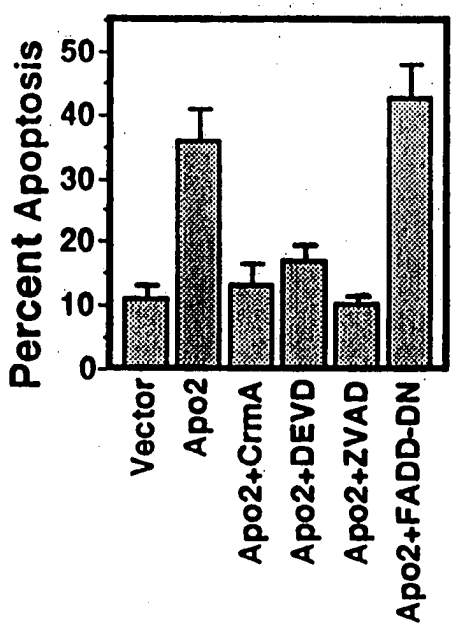
4A



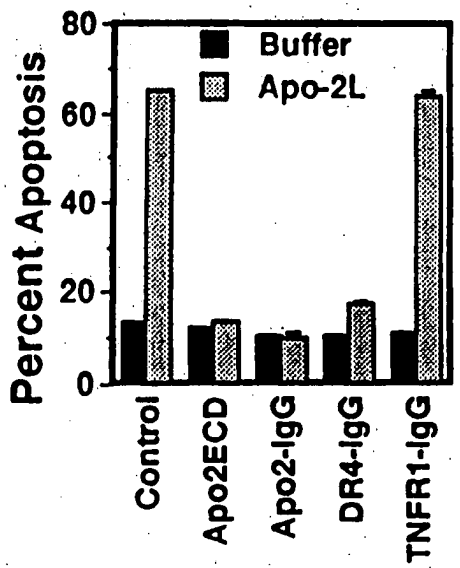
4B



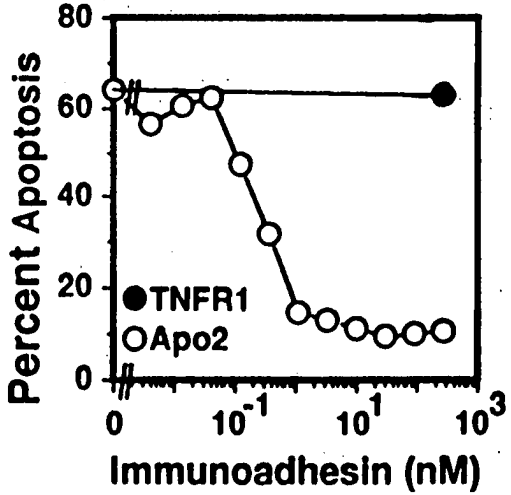
4C



4D



4E



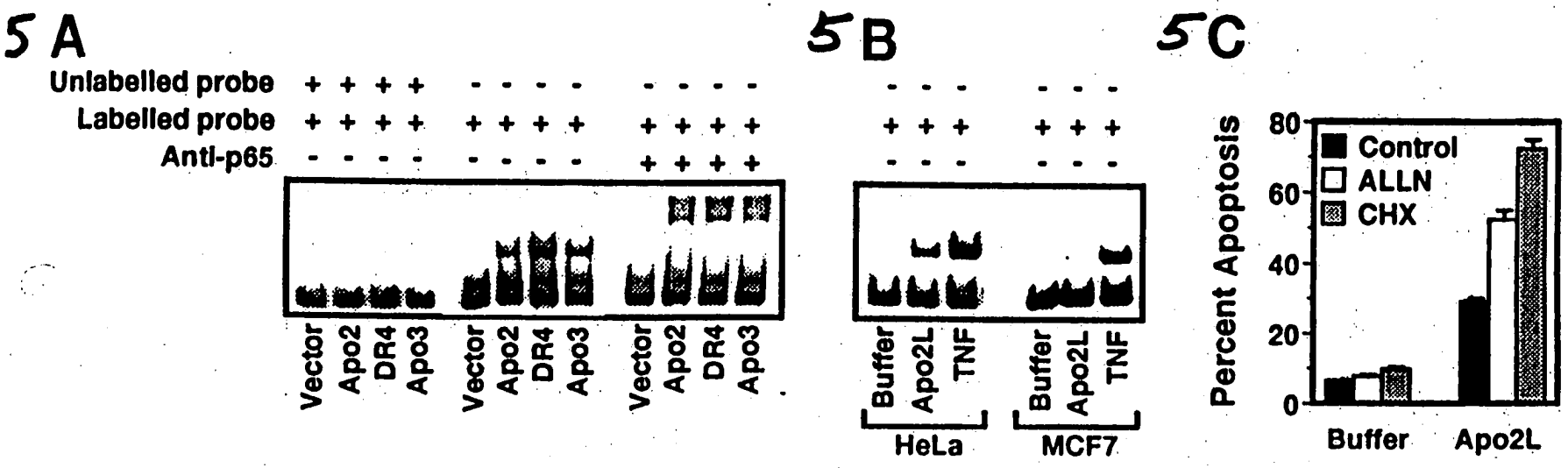

5A
Unlabelled probe + + + + - - - - - - - -
Labelled probe + + + + + + + + + + + +
Anti-p65 - - - - - - - - + + + +
Vector
Apo2
DR4
Apo3
Vector
Apo2
DR4
Apo3
Vector
Apo2
DR4
Apo3
5B
Buffer
Apo2L
TNF
Buffer
Apo2L
TNF
HeLa
MCF7
5C
Percent Apoptosis
80
60
40
20
0
Control
ALLN
CHX
Buffer
Apo2L

FIG. 5

Fetal
kidndey
liver
lung
brain
Adult
PBL
colon
sm. intest.
ovary
testis
prostate
thymus
spleen
pancreas
kidndey
sk. muscle
liver
lung
placenta
brain
heart

FIG. 6

NOVEL POLYPEPTIDES WITHIN THE TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY AND USES THEREFOR

Background of the Invention

In multicellular organisms, homeostasis is maintained by balancing the rate of cell proliferation against the rate of cell death. This balance is important in pathophysiologic contexts (for example, in the elimination of virally-infected and radiation-damaged cells). Cell proliferation is influenced by numerous growth factors and the expression of proto-oncogenes, which typically encourage progression through the cell cycle. In contrast, numerous events, including the expression of tumor suppressor genes, can lead to an arrest of cellular proliferation.

In differentiated cells, a particular form of cell death called apoptosis (or programmed cell death (PCD)) is carried out when an internal suicide program is activated. This program can be initiated by a variety of external signals as well as signals that are generated within the cell in response to, for example, genetic damage. Thus, apoptosis of a cell or a group of cells is presumably beneficial to the organism as a whole. For many years, the magnitude of apoptotic cell death was not appreciated because the dying cells are quickly eliminated by phagocytes, without an inflammatory response.

The mechanisms that mediate apoptosis have been intensively studied. These mechanisms involve the activation of endogenous proteases, loss of mitochondrial function, and structural changes such as disruption of the cytoskeleton, cell shrinkage, membrane blebbing, and nuclear condensation, which occurs as the cell's DNA is degraded. Initially, large fragments of DNA (of about 50 kb) are

homologous from one family member to another vary. Two family members may have homologous sequence in the ectodomain, but not in the death domain, or vice-versa.

The death domain of the Fas/APO-1 receptor interacts with FADD (Fas-associating protein with death domain, also known as MORT1) and RIP (receptor interacting protein), forming a complex that, when joined by Caspase-8, constitutes the Fas/APO-1 death-inducing signalling complex (Boldin et al., *supra*; Muzio et al., *supra*). The interaction between Fas/APO-1 and FADD is mediated by their respective C-terminal death domains (Chinnaiyan et al., *Cell* 81:505-512, 1995).

A second complex that is thought to be involved in cell death forms in association with the intracellular portion of TNFR-1, and includes Caspase-8, TRADD (TNFR-1-associated death domain protein), and FADD/MORT1 (Boldin et al.,*supra*; Muzio et al., *supra*).

Just as not all members of the TNF receptor family bind TNF (see above), not all members contain a death domain. For example, a receptor termed TNFR-2 is a 75 kDa receptor for the TNF ligand that is not believed to contain a death domain. Thus, this receptor may activate an alternative intracellular signalling pathway that may or may not lead to apoptosis (WO 96/34095; Smith et al., *Cell* 76:959-962, 1994).

The factors that are known to bind TNFR-1 include TNF-α and TNF-β (also known as lymphotoxin-α), which are related members of a broad family of polypeptide mediators, collectively known as cytokines, that includes the interferons, interleukins, and growth factors (Beutler and Cerami, *Ann. Rev. Immunol.*, 7:625-655, 1989). A subset of these polypeptides are classified as TNF-related cytokines

and, in addition to TNF-α and TNF-β, include LT-β and ligands for the Fas and 4-1BB receptors.

TNF-α and TNF-β were first recognized for their anti-tumor activities, but are now known as pleiotropic cytokines that play a role in many biological processes. For example, TNF-α is believed to mediate immunostimulation, autoimmune disease, graft rejection, anti-viral responses, septic shock, cerebral malaria, cytotoxicity, protective responses to ionizing radiation, and growth regulation. TNF-β, which is produced by activated lymphocytes, exhibits similar but not identical biological activities. TNF-β elicits tumor necrosis, mediates anti-viral responses, activates polymorphonuclear leukocytes, and induces the expression of MHC class I antigens and adhesion molecules on endothelial cells.

Summary of the Invention

The present invention relates to the discovery and characterization of two novel polypeptides with similarity to members of the TNF receptor superfamily. The first, Tango-63d, is a 440 amino acid polypeptide, and the second, Tango-63e, is a 411 amino acid polypeptide that is identical to Tango-63d, with the exception of a deletion of amino acids 183-211.

The invention encompasses nucleic acid molecules encoding Tango-63d and Tango-63e, vectors containing these nucleic acid molecules, cells harboring recombined DNA encoding Tango-63d and/or Tango-63e, fusion proteins that include Tango-63d and/or Tango-63e, transgenic animals that express Tango-63d and/or Tango-63e, and recombinant knock-out animals that fail to express Tango-63d and/or Tango-63e.

By "isolated nucleic acid molecule" is meant a nucleic acid molecule that is separated from either the

chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. The polypeptide can be a naturally occurring, synthetic, or a recombinant molecule consisting of a hybrid with one portion, for example, being encoded by all or part of a Tango-63 gene, and a second portion being encoded by all or part of a second gene. For example, the polypeptide can be fused to a hexa-histidine tag to facilitate purification of bacterially expressed protein, or to a hemagglutinin (HA) tag to facilitate purification of protein expressed in eukaryotic cells. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., *Cell* 37:767, 1984). The polypeptides of the invention can also be fused to another compound (such as polyethylene glycol) that will increase the half-life of the polypeptide within the circulation. Similarly, the receptor polypeptide can be fused to a heterologous polypeptide such as the Fc region of an IgG molecule, or a leader or secretory sequence.

The polypeptides of the invention can be chemically synthesized, produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect, and mammalian cells in culture), or they can be purified from tissues in which they are naturally expressed, according to standard biochemical methods of purification.

The polypeptides of the present invention can be employed to identifying putative ligands to which the polypeptides bind. These ligands can be identified, for example, by transfecting a cell population with an appropriate vector from which the polypeptide is expressed, and exposing that cell to various putative ligands. The ligands tested could include those that are known to interact with members of the TNF receptor superfamily, as

well as additional small molecules, cell supernatants, extracts, or other natural products. The polypeptide can also be used to screen an expression library according to standard techniques. This is not to say that the polypeptides of the invention must interact with another molecule in order to exhibit biological activity; the polypeptides may function in a ligand-independent manner. [redacted] of the invention using no more than routine pharmacological assays.

Also included in the invention are "functional polypeptides," which possess one or more of the biological functions or activities of Tango-63d or Tango-63e. These functions or activities are described in detail below and concern, primarily, [redacted] by, for example, binding some or all of the proteins which normally bind to Tango-63d or Tango-63e. A functional polypeptide is also considered within the scope of the invention if it serves as an immunogen for production of antibodies that specifically bind to Tango-63d or Tango-63e. In many cases, functional polypeptides retain one or more domains present in the naturally-occurring form of the polypeptide. For example, a functional polypeptide can posses one or more of the Tango-63 domains, for example, an extracellular domain, a transmembrane domain, and an intracellular domain. It is well within the abilities of skilled artisans to determine whether a polypeptide, regardless of size, retains the functional activity of a polypeptide of the invention.

The functional polypeptides can contain a primary amino acid sequence that has been modified from those disclosed herein. Preferably these modifications consist of conservative amino acid substitutions, as described herein.

When the polypeptides of the invention are administered to a patient, they may be given in a membrane-bound or a soluble, circulating form. Typically, the soluble form of the polypeptide will lack the transmembrane domain. Soluble polypeptides may include any number of leader sequences at the 5' end; the purpose of these leader sequences being, primarily, to allow expression in a eukaryotic system (see, for example, U.S. Patent No. 5,082,783).

The members of a pair of molecules (for example, an antibody-antigen pair or a receptor-ligand pair) are said to "specifically bind" to each other if they bind to each other with greater affinity than to other molecules, even those that are structurally or functionally related to a member of the specific binding pair.

The invention also encompasses compounds which modulate the expression or activity of Tango-63d and/or Tango-63e, including small molecules (i.e., molecules with a molecular weight below about 500), large molecules (i.e., molecules with a molecular weight above about 500), and nucleic acid molecules that can be used to inhibit the expression of these genes (for example, antisense and ribozyme molecules) or to enhance their expression (for example, expression constructs that place nucleic acid sequences encoding either Tango-63d or Tango-63e under the control of a strong promoter system), and transgenic animals that express a Tango-63 transgene.

Tango-63d and/or Tango-63e function can be altered either by altering the expression of Tango-63d and/or Tango-63e (i.e., altering the amount of Tango-63d and/or Tango-63e present in a given cell) or by altering the activity of Tango-63d and/or Tango-63e.

The invention includes methods for treating disorders characterized by aberrant expression or activity

of Tango-63d and/or Tango-63e. These methods entail administering a molecule which increases or decreases, as appropriate, expression of Tango-63d and/or Tango-63e.

The invention encompasses methods of treatment including a method of treating a patient who has a disorder associated with an abnormal rate of apoptotic cell death by administering a compound that modulates the expression of Tango-63d and/or Tango-63e (at the DNA, mRNA or protein level, e.g., by altering mRNA splicing) or the activity of Tango-63d and/or Tango-63e. Examples of such compounds include small molecules, antisense nucleic acid molecules, ribozymes, and molecules that specifically interact with the polypeptide and thereby act as full or partial agonists or antagonists of its activity.

Disorders that can be treated by altering the expression or activity of the polypeptides of the invention include disorders associated with either an abnormally high or an abnormally low rate or apoptotic cell death (as described further hereinbelow). In addition, T cell mediated diseases, including acquired immune deficiency syndrome (AIDS), autoimmune diseases such as systemic lupus erythrematosis, rheumatoid arthritis, and type I diabetes, septic shock, cerebral malaria, graft rejection, cytotoxicity, cachexia, and inflammation are considered amenable to treatment by altering the expression or activity of a polypeptide of the invention.

A patient who has a disorder associated with an abnormally high rate of apoptotic cell death can be treated by the administration of: a ligand (for example, a naturally occurring or synthetic ligand) that antagonizes Tango-63d or Tango-63e; a compound that decreases the expression of Tango-63d or Tango-63e; a compound that decreases the activity of Tango-63d or Tango-63e; an

expression vector that contains a nucleic acid molecule that encodes a nonfunctional Tango-63; or a nonfunctional Tango-63 polypeptide itself. Preferably, the nonfunctional polypeptide will bind any naturally occurring ligand(s) of Tango-63d or Tango-63e or otherwise interfere with the ability of the polypeptides to transduce a signal. Accordingly, the invention features therapeutic compositions that contain the compounds or ligands described above.

Conversely, a patient who has a disorder associated with an abnormality-low-rate-of-apoptoric-cell-death-can-be treated by the administration of a ligand (for example, a naturally occurring or synthetic ligand) that activates Tango-63d or Tango-63e (i.e., a ligand that acts as a full or partial agonist of Tango-63d or Tango-63e); a compound that increases the expression of Tango-63d or Tango-63e; a compound that increases the activity of Tango-63d or Tango-63e; an expression vector that contains a nucleic acid molecule encoding Tango-63d or Tango-63e, or by administering either or both of the polypeptides directly to the patient's cells (either *in vivo* or *ex vivo*). These methods are described more fully below.

Certain disorders are associated with an increased number of surviving cells, which are produced and continue to survive or proliferate when apoptosis is inhibited. These disorders include cancer, particularly follicular lymphomas, carcinomas associated with mutations in p53, and hormone-dependent tumors such as breast cancer, prostate cancer, and ovarian cancer. As described in the example below, Tango-63 has been mapped to a position that is located in the most frequently lost region of chromosome 8, between markers D8S133 and NEFL. As described in the example below, this region has been implicated in the etiology of numerous cancers, including prostate cancer,

colon cancer, non-small cell lung cancer, breast cancer, head and neck cancer, hepatocarcinoma, and bladder cancer.

Additional disorders that are associated with an increased number of surviving cells include autoimmune disorders (such as systemic lupus erythematosus and immune-mediated glomerulonephritis), and viral infections (such as those caused by herpesviruses, poxviruses, and adenoviruses).

Populations of cells are often depleted in the event of viral infection, with perhaps the most dramatic example being the cell depletion caused by the human immunodeficiency virus (HIV). Surprisingly, most T cells that die during HIV infections do not appear to be infected with HIV. Although a number of explanations have been proposed, recent evidence suggests that stimulation of the CD4 receptor results in the enhanced susceptibility of uninfected T cells to undergo apoptosis.

A wide variety of neurological diseases are characterized by the gradual loss of specific sets of neurons. Such disorders are referred to as neurodegenerative diseases and include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), Huntington's disease, retinitis pigmentosa, spinal muscular atrophy, and various forms of cerebellar degeneration. The cell loss in these diseases does not induce an inflammatory response, and apoptosis appears to be the mechanism of cell death.

In addition, a number of hematologic diseases are associated with a decreased production of blood cells. These disorders include anemia associated with chronic disease, aplastic anemia, chronic neutropenia, and the myelodysplastic syndromes. Disorders of blood cell production, such as myelodysplastic syndrome and some forms

of aplastic anemia, are associated with increased apoptotic cell death within the bone marrow. These disorders could result from the activation of genes that promote apoptosis, acquired deficiencies in stromal cells or hematopoietic survival factors, or the direct effects of toxins and mediators of immune responses.

Two common disorders associated with cell death are myocardial infarction (which is commonly referred to as a "heart attack") and cerebral ischemia (which is commonly referred to as "stroke"). In both of these disorders, cells within the central area of ischemia, which is produced in the event of acute loss of blood flow, appear to die rapidly as a result of necrosis. However, outside the central ischemic zone, cells die over a more protracted time period and, morphologically, appear to die by apoptosis.

The present invention encompasses methods and compositions for the diagnostic evaluation, typing, and prognosis of disorders associated with apoptotic cell death and disorders related to abnormal expression or activity or Tango-63d or Tango-63e. The disorder can be associated with either an increase or a decrease in the incidence of apoptotic cell death. For example, the nucleic acid molecules of the invention can be used as diagnostic hybridization probes to detect, for example, expression of Tango-63d or Tango-63e. Such methods can be used to classify cells by their level of Tango-63d or Tango-63e expression. ~~For example, higher Tango-63d or Tango-63e expression may be associated with a higher rate~~ of apoptosis. The present invention further provides for diagnostic kits for the practice of such methods.

In particular, the invention described below encompasses Tango-63d or Tango-63e polypeptides corresponding to functional domains of Tango-63d or

Tango-63e (e.g., the death domain), mutated, truncated, or deleted polypeptides that retain at least one of the functional activities of Tango-63d or Tango-63e (for example, a polypeptide in which one or more amino acid residues have been substituted, deleted from, or added to the death domain without destroying the ability of the mutant Tango-63d or Tango-63e polypeptides to induce apoptosis, and fusion proteins as described below).

Polypeptides that exhibit at least 70%, preferably at least 80%, more preferably at least 90%, and most preferably at least 95% of the activity of the Tango-63d or Tango-63e polypeptides described herein are considered within the scope of the invention.

The invention encompasses nucleic acids and polypeptides that have a sequence that is substantially identical to a Tango-63d or Tango-63e nucleic acid or polypeptide. The term "substantially identical" refers to a polypeptide or nucleic acid having a sequence that is at least 85%, preferably at least 90%, more preferably at least 95%, and most preferably at least 98% or 99% or more identical to the sequence of a reference amino acid or nucleic acid sequence. For polypeptides, the length of the reference polypeptide sequence will generally be at least 16 amino acids, at least 20 amino acids, at least 25 amino acids, or preferably at least 35 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least 50 nucleotides, at least 60 nucleotides, at least 75 nucleotides, or at least 90 nucleotides.

Sequence identity can be measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin

Biotechnology Center, 1710 University Avenue, Madison, WI 53705) with the default parameters specified therein.

In the case of polypeptide sequences that are less than 100% identical to a reference sequence, the non-identical positions are preferably, but not necessarily, conservative substitutions for the reference sequence. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

Where a particular polypeptide is said to have a specific percent identity to a reference polypeptide of a defined length, the percent identity is relative to the reference polypeptide. Thus, a peptide that is 50% identical to a reference polypeptide that is 100 amino acids long can be a 50 amino acid polypeptide that is completely identical to a 50 amino acid long portion of the reference polypeptide. It might also be a 100 amino acid long polypeptide which is 50% identical to the reference polypeptide over its entire length. Of course, many other polypeptides will meet the same criteria.

The reference nucleic acid or polypeptide can be a naturally-occurring molecule, for example, a Tango-63d-encoding nucleic acid molecule, a Tango-63e-encoding nucleic acid molecule, a Tango-63d polypeptide, or a Tango-63e polypeptide.

A transgenic animal is any animal containing cells that bear genetic information received, directly or indirectly, by deliberate genetic manipulation at the subcellular level, such as DNA received by microinjection or by infection with recombinant virus. Thus, animals of the invention are those with one or more cells that contain a

recombinant DNA molecule of the invention and, in this context, the term "animal" denotes all animals except *Homo sapiens*. Farm animals (pigs, goats, sheep, cows, horses, rabbits, and the like), rodents (such as rats, guinea pigs, and mice), non-human primates (for example, baboons, monkeys, and chimpanzees), and domestic animals (for example, dogs and cats) are especially preferred.

It is also preferred that the nucleic acid molecule becomes integrated with the animal's chromosomes, but the use of DNA sequences that replicate extrachromosomally, such as might be engineered into yeast artificial chromosomes (YACs) or human artificial chromosomes (HACs), are also contemplated.

Transgenic animals include animals in which the genetic information has been taken up and integrated into a germ line cell. These animals typically have the ability to transfer the genetic information to their offspring. If the offspring in fact possess some or all of the genetic information delivered to the parent animal, then they, too, are transgenic animals.

In another embodiment, the invention features methods of identifying compounds that modulate apoptotic cell death by modulating the expression or activity of Tango-63d and/or Tango-63e by assessing the expression or activity of Tango-63d and/or Tango-63e in the presence and absence of the compound. A difference in the level of expression or activity of Tango-63d or Tango-63e in the presence of the compound (compared with the level of expression or activity in the absence of the compound) indicates that the compound is capable of modulating the expression or activity of Tango-63d or Tango-63e and thereby useful in, for example, modulating apoptotic cell death. Expression can be assessed either at the level of gene

expression (e.g., by measuring mRNA) or protein expression by techniques that are well known to skilled artisans. The activity of Tango-63d or Tango-63e can be assessed functionally, i.e., by assaying the ability of the compound to inhibit apoptosis following activation of the Tango-63d or Tango-63e receptor complexes.

The invention features an isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide that is at least 85% identical to SEQ ID NO:2; and an isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide that is at least 85% identical to SEQ ID NO:4.

In other aspect, the invention features: an isolated nucleic acid molecule that includes the nucleotide sequence of SEQ ID NO:1, and that encodes the amino acid sequence of SEQ ID NO:2; an isolated nucleic acid molecule that includes the nucleotide sequence of SEQ ID NO:3, and that encodes the amino acid sequence of SEQ ID NO:4; an isolated nucleic acid molecule that includes the molecule deposited with the American Type Culture Collection and assigned accession number 98368; and an isolated nucleic acid molecule that includes the molecule deposited with the American Type Culture Collection and assigned accession number 98367.

In another aspect, the invention features a vector that includes an above-described nucleic acid molecule. In various specific embodiments, the vector is an expression vector, and can include a regulatory element such as the cytomegalovirus hCMV immediate early gene, the early promoter of SV40 adenovirus, the late promoter of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage λ, the control regions of fd coat protein, the

promoter for 3-phosphoglycerate kinase, the promoters of acid phosphatase, and the promoters of the yeast α-mating factors. The vector can also include a regulatory element that directs tissue-specific expression, a reporter gene such as a gene encoding β-lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo^r, G418^r), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), lacZ (encoding β-galactosidase), and xanthine guanine phosphoribosyltransferase (XGPRT). The vector can be a plasmid or a virus, such as a retrovirus.

In another aspect, the invention features a genetically engineered host cell, particularly a eukaryotic cell, which includes a vector, as described above.

In another aspect, the invention features a chimeric polypeptide that contains a polypeptide encoded by one or more of the nucleic acid molecules described above and a heterologous polypeptide (i.e. a polypeptide that has a sequence other than those described above as polypeptides of the invention).

In other aspects, the invention features an antibody that specifically binds Tango-63d and an antibody that specifically binds Tango-63e.

In yet another aspect, the invention features a transgenic animal harboring a nucleic acid molecule described above.

The invention also features a method for determining whether a patient has a disorder associated with an abnormal rate of apoptotic cell death. The method is carried out by quantitating the level of expression of Tango-63d or Tango-63e in a biological sample (e.g., a tumor sample) obtained from the patient. Expression can be assessed by

examining the level of mRNA encoding Tango-63d or Tango-63e or the level of Tango-63d or Tango-63e protein. Methods of quantitating mRNA and protein are well known in the art of molecular biology. Methods useful in the present invention include RNAse protection assays, Northern blot analyses, the polymerase chain reaction (PCR, particularly, RT-PCR), and, to assess the level of protein expression, Western blot analyses.

The invention also features a method for determining whether a patient has a disorder associated with a mutation in a gene encoding Tango-63d or Tango-63e. The method is carried out by examining the nucleic acid sequence of Tango-63d or Tango-63e in a sample of DNA obtained from a patient.

The invention also features a method of treating a patient who has a disorder associated with abnormal activity of the Tango-63d or Tango-63e complex. The method is carried out by administering to the patient a compound that modulates the expression or activity of Tango-63d or Tango-63e. The compound can be, for example, a compound that acts as a full or partial agonist of Tango-63d or Tango-63e (which would be administered to increase the activity of Tango-63d or Tango-63e) or as a full or partial antagonist of Tango-63d or Tango-63e (which would be administered to decrease the activity of Tango-63d or Tango-63e). The compound could be a small molecule. To decrease the expression of Tango-63d or Tango-63e, an antisense nucleic acid molecule, or a ribozyme can be administered.

The invention also features therapeutic compositions which include the compounds that are used in the methods of treatment described above. The compounds identified as useful can be naturally occurring or synthetic.

In another aspect, the invention features a method for treating a patient who has a disorder associated with abnormal activity of the Tango-63d or Tango-63e by administering to the patient a compound that mediates oligomerization between Tango-63d or Tango-63e and other molecules that may assemble to form an active complex. These molecules can include TRADD, MORT1, and Caspase-8, or homologues thereof.

The patient who is treated can have any disorder associated with an abnormal level of apoptotic cell death, including acquired immune deficiency syndrome (AIDS), a neurodegenerative disorder, a myelodysplastic syndrome, an ischemic injury, a toxin-induced injury, or a cancer.

The invention also features a method of treating a patient who has a disorder associated with excessive apoptotic cell death by administering to the patient Tango-63d and/or Tango-63e nucleic acid molecules or the Tango-63d and/or Tango-63e polypeptides.

In another aspect, the invention features a method of identifying a compound that modulates expression of Tango-63d and/or Tango-63e by assessing the expression of Tango-63d or Tango-63e in the presence and absence of the compound.

The invention also features a method of treating a patient who has an abnormally low rate of apoptotic cell death. The method is carried out by administering to the patient a compound that mediates oligomerization between Tango-63d and/or Tango-63e and intracellular polypeptides that interact with Tango-63d or Tango-63e to transduce an apoptotic signal that leads to the cell's death.

The invention also features a method of identifying a compound that modulates the activity of Tango-63d and/or

Tango-63e by assessing the activity of Tango-63d and/or Tango-63e in the presence and absence of the compound.

In other aspects, the invention includes a method for determining whether a compound modulates oligomerization between Tango-63d and/or Tango-63e and polypeptides that form a complex with these polypeptides by examining oligomerization of Tango-63d and/or Tango-63e and these polypeptides in the presence and absence of the compound. An administered compound may modulate oligomerization between and Tango-63d or Tango-63e and, for example, Caspase-8 or Caspase-8-like polypeptides, TRADD or TRADD-like polypeptides, and FADD/MORT-1 or FADD-MORT-1-like polypeptides.

The invention features an isolated nucleic acid molecule that hybridizes under stringent conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, the isolated nucleic acid molecule encoding Tango-63d; an isolated nucleic acid molecule that hybridizes under stringent conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:3, the isolated nucleic acid molecule encoding Tango-63e; an isolated nucleic acid molecule that includes a nucleotide sequence that is at least 90% identical to the nucleotide sequence of SEQ ID NO:1, the isolated nucleic acid molecule encoding Tango-63d; and an isolated nucleic acid molecule that includes a nucleotide sequence which is at least 90% identical to the nucleotide sequence of SEQ ID NO:3, the isolated nucleic acid molecule encoding Tango-63e.

Also considered within the scope of the invention is a nucleic acid molecule that: hybridizes under stringent conditions to cDNA sequence contained within ATCC Accession No. 98367; hybridizes under stringent conditions to cDNA sequence contained within ATCC Accession No. 98368; is 85%

identical to SEQ ID NO:1 (Fig. 1); is 85% identical to SEQ ID NO:3 (Fig. 2); is 95% identical to SEQ ID NO:1; is 95% identical to SEQ ID NO:3; is 85% identical to cDNA sequence contained within ATCC Accession No. 98367; is 85% identical to cDNA sequence contained within ATCC Accession No. 98368; is 95% identical to cDNA sequence contained within ATCC Accession No. 98367; is 95% identical to cDNA sequence contained within ATCC Accession No. 98368; hybridizes under stringent conditions to nucleotides 128 to 1447 of SEQ ID NO:1 (Fig. 1); or hybridizes under stringent conditions to nucleotides 128 to 1360 of SEQ ID NO:3 (Fig. 2). Polypeptides encoded by these nucleic acids are also considered within the scope of the invention.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

Other features and advantages of the invention will be apparent from the detailed description and from the claims. Although materials and methods similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred materials and methods are described below.

Brief Description of the Drawings

Fig. 1 is a representation of the nucleic acid sequence of Tango-63d (SEQ ID NO:1 (bottom)) and the amino acid sequence of the polypeptide it encodes (SEQ ID NO:2 (top)).

Fig. 2 is a representation of the nucleic acid sequence of Tango-63e (SEQ ID NO:3 (bottom)) and the amino acid sequence of the polypeptide it encodes (SEQ ID NO:4 (top)).

Detailed Description

The present invention relates to the discovery, identification, and characterization of two nucleic acid molecules that encode novel polypeptides, *i.e.*, Tango-63d and Tango-63e.

Nucleic Acid Molecules of the Invention

Isolated nucleic acid molecules, as defined above, can be cDNA, genomic DNA, synthetic DNA, or RNA, and can be double-stranded or single-stranded (*i.e.*, either a sense or an antisense strand). Fragments of these molecules, which are also considered within the scope of the invention, can be produced, for example, by the polymerase chain reaction (PCR) or generated by treatment with one or more restriction endonucleases. A ribonucleic acid (RNA) molecule can be produced by *in vitro* transcription.

The nucleic acid molecules of the invention can contain naturally occurring sequences, or sequences that differ from those that occur naturally, but, due to the degeneracy of the genetic code, encode the same polypeptide. In addition, these nucleic acid molecules are not limited to sequences that only encode functional polypeptides, and thus, can include coding sequence that encodes a

nonfunctional polypeptide, as well as some or all of the non-coding sequences that lie upstream or downstream from a coding sequence.

The nucleic acid molecules of the invention can be synthesized (for example, by phosphoramidite-based synthesis) or obtained from a biological cell, such as the cell of a mammal. Thus, the nucleic acids can be those of a human, mouse, rat, guinea pig, cow, sheep, horse, pig, rabbit, monkey, dog, or cat. Combinations or modifications of the nucleotides within these types of nucleic acids are also encompassed.

The isolated nucleic acid molecules of the invention encompass fragments that are not found as such in the natural state. Thus, the invention encompasses recombinant molecules, such as those in which a nucleic acid sequence (for example, a sequence encoding Tango-63d or Tango-63e) is incorporated into a vector (for example, a plasmid or viral vector) or into the genome of a heterologous cell (or the genome of a homologous cell, at a position other than the natural chromosomal location). These circumstances are discussed further below.

In the event the nucleic acid molecules of the invention encode or act as antisense molecules, they can be used for example, to regulate transcription of the nucleic acid molecules of the invention. With respect to regulation of Tango-63d or Tango-63e transcription, such techniques can be used to diagnose and/or treat disorders associated with apoptotic cell death. These nucleic acids will be discussed further in that context.

In addition to the nucleotide sequences disclosed herein (see, for example SEQ ID NOs:1 and 3), equivalent forms can be present in other species, and can be identified and isolated by using the nucleotide sequences disclosed

herein and standard molecular biological techniques. For example, homologs of Tango-63d and Tango-63e can be isolated from other organisms by performing PCR using two degenerate oligonucleotide primer pools designed on the basis of amino acid sequences that are conserved in Tango-63d and Tango-63e. Alternatively, the method used to identify human Tango-63d and Tango-63e can be used to isolate homologs from other species. The template for the reaction can be cDNA obtained by reverse transcription of mRNA prepared from, for example, human or non-human cell lines or tissues, particularly those known or suspected to express Tango-63d or Tango-63e (see the expression data presented in the example below). The PCR product can be subcloned and sequenced to ensure that the amplified nucleic acid sequence represents the sequence of Tango-63d or Tango-63e. Once identified, Tango-63d and Tango-63e in other species can be used, in turn, to develop animal models for the purpose of drug discovery. Alternatively, these members of the TNF receptor superfamily can be used in *in vitro* assays for the purpose of drug discovery.

The invention also encompasses nucleotide sequences that encode mutant Tango-63d or Tango-63e, or fragments thereof, that retain one or more functions of Tango-63d or Tango-63e, as described herein.

The invention also encompasses: (a) expression vectors that contain any of the foregoing Tango-63d or Tango-63e coding sequences and/or their complements (that is, "antisense" sequence); (b) expression vectors that contain Tango-63d or Tango-63e coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences; (c) expression vectors containing Tango-63d or Tango-63e nucleic acid molecules and heterologous nucleic acid molecules, such as molecules

encoding a reporter or marker; and (d) genetically engineered host cells that contain any of the foregoing expression vectors and thereby express the nucleic acid molecules of the invention in the host cell.

As used herein, regulatory elements include but are not limited to inducible and non-inducible promoters, enhancers, operators and other elements, which are known to those skilled in the art, that drive and regulate gene expression. Such regulatory elements include but are not limited to the cytomegalovirus hCMV immediate early gene, the early or late promoters of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage A, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase, the promoters of acid phosphatase, and the promoters of the yeast α-mating factors.

Similarly, the nucleic acid can form part of a hybrid gene encoding additional polypeptide sequences (for example, sequences that function as a marker or reporter) that can be used, for example, to produce a fusion protein (as described further below). Examples of marker or reporter genes include β-lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo^r, $G418^r$), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), lacZ (encoding β-galactosidase), and xanthine guanine phosphoribosyltransferase (XGPRT). As with many of the standard procedures associated with the practice of the invention, skilled artisans will be aware of additional useful reagents, for example, of additional sequences that can serve the function of a marker or reporter.

The expression systems that can be used for purposes of the invention include but are not limited to microorganisms such as bacteria (for example, *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the nucleic acid molecules of the invention; yeast (for example, *Saccharomyces* and *Pichia*) transformed with recombinant yeast expression vectors containing the nucleic acid molecules of the invention (preferably containing the nucleic acid sequences of Tango-63d and/or Tango-63e); insect cell systems infected with recombinant virus expression vectors (for example, baculovirus) containing the nucleic acid molecules of the invention; plant cell systems infected with recombinant virus expression vectors (for example, cauliflower mosaic virus (CamV) and tobacco mosaic virus (TMV)) or transformed with recombinant plasmid expression vectors (for example, Ti plasmid) containing Tango-63d and/or Tango-63e nucleotide sequences; or mammalian cell systems (for example, COS, CHO, BHK, 293, VERO, HeLa, MDCK, WI38, and NIH 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (for example, the metallothionein promoter) or from mammalian viruses (for example, the adenovirus late promoter and the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors can be advantageously selected depending upon the use intended for the gene product being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of Tango-63d or Tango-63e polypeptides for raising antibodies to those polypeptides, vectors that are capable of directing the expression of high levels of fusion protein products that are readily purified can be desirable. Such vectors

include, but are not limited to, the *E. coli* expression vector pUR278 (Ruther et al., *EMBO J.* 2:1791, 1983), in which the coding sequence of the insert can be ligated individually into the vector in frame with the lacZ coding region so that a fusion protein is produced; pIN vectors (Inouye and Inouye, *Nucleic Acids Res.* 13:3101-3109, 1985; Van Heeke and Schuster, *J. Biol. Chem.* 264:5503-5509, 1989); and the like. pGEX vectors can also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The coding sequence of the insert can be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *S. frugiperda* cells in which the inserted gene is expressed. (for example, see Smith et al. *J. Virol.* 46:584, 1983; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems can be utilized. In cases where an

fashion desired. Such modifications (for example, glycosylation) and processing (for example, cleavage) of protein products can be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product can be used.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express Tango-63d or Tango-63e sequences described above can be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (for example, promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells can be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method can advantageously be used to engineer cell lines which produce Tango-63d and/or Tango-63e. Such engineered cell lines can be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the gene product.

A number of selection systems can be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., *Cell* 11:223, 1977), hypoxanthine-guanine phosphoribosyltransferase (Szybalska and Szybalski, *Proc. Natl. Acad. Sci. USA* 48:2026, 1962), and adenine phosphoribosyltransferase (Lowy, et al., *Cell* 22:817, 1980) genes can be employed in tk$^-$, hgprt$^-$ or aprt$^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: *dhfr*, which confers resistance to methotrexate (Wigler, et al., *Proc. Natl. Acad. Sci. USA* 77:3567, 1980; O'Hare, et al., *Proc. Natl. Acad. Sci. USA* 78:1527, 1981); *gpt*, which confers resistance to mycophenolic acid (Mulligan and Berg, *Proc. Natl. Acad. Sci. USA* 78:2072, 1981); *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., *J. Mol. Biol.* 150:1, 1981); and *hygro*, which confers resistance to hygromycin (Santerre et al., *Gene* 30:147, 1984).

Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (*Proc. Natl. Acad. Sci. USA* 88:8972-8976, 1991). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+} nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

Polypeptides of the Invention

The Tango-63d and Tango-63e polypeptides described herein and fragments, mutants, and truncated forms thereof, including fusion proteins, can be prepared for a variety of uses, including but not limited to the generation of antibodies, as reagents in diagnostic assays, for the identification of other cellular gene products involved in the regulation of apoptosis, as reagents in assays for screening for compounds that can be used in the treatment of disorders associated with apoptotic cell death, or abnormal activity of polypeptides in the TNF receptor superfamily, and as pharmaceutical reagents useful in the treatment of such disorders.

The invention encompasses proteins and polypeptides that have one or more of the functions of naturally-occurring Tango-63d or Tango-63e. The functional attributes of Tango-63d and Tango-63e may include one or more of the following: the ability to bind TRADD, and the ability to initiate a biochemical reaction that induces apoptosis. Polypeptides having one or more functions of naturally-occurring Tango-63d or Tango-63e (i.e., functionally equivalent polypeptides) can include, but are not limited to, polypeptides that contain additions or substitutions of amino acid residues within sequences encoded by the nucleic acid molecules described above (see SEQ ID NOs:1 and 3), or that are encoded by nucleic acid molecules which result in a silent change, and thus produce a functionally equivalent gene product. Amino acid substitutions can be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. Amino acids that are typically considered as providing a conservative

stringent conditions to a nucleic acid molecule having the sequence of SEQ ID NO: 1; polypeptides encoded by nucleic acid molecules which hybridize under stringent conditions to a nucleic acid molecule having the sequence of SEQ ID NO:3; polypeptides encoded by nucleic acid molecules which hybridize under stringent conditions to a nucleic acid molecule having the sequence of the Tango-63d encoding portion of the clone designated ATCC accession number 98368; and polypeptides encoded by nucleic acid molecules which hybridize under stringent conditions to a nucleic acid molecule having the sequence of the Tango-63e encoding portion of the clone designated ATCC accession number 98367.

Antibodies

The invention also encompasses antibodies that bind Tango-63d or Tango-63e. Antibodies that specifically recognize one or more epitopes of these proteins, or fragments thereof are also encompassed by the invention. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

The antibodies of the invention can be used, for example, in the detection of various forms of Tango-63d or Tango-63e in a biological sample and can, therefore, be utilized as part of a diagnostic or prognostic technique whereby patients can be tested for abnormal amounts of Tango-63d or Tango-63e. Such antibodies can also be utilized in conjunction with, for example, compound screening schemes, as described below, for the evaluation of the effect of test compounds on expression and/or activity of Tango-63d or Tango-63e. Additionally, such antibodies

can be used in conjunction with the gene therapy techniques described below, to, for example, evaluate cells expressing the alternate forms described herein prior to their introduction into the patient. Preferably, the antibodies recognize epitopes of Tango-63d or Tango-63e that are unique, i.e., are not present on related molecules, such as members of the TNF receptor superfamily (e.g., TNFR-1) or more distantly related proteins. Accordingly, the antibodies are preferably raised against a peptide sequence present in Tango-63d or Tango-63e that is not present in related molecules, such as members of the TNF receptor superfamily.

For the production of antibodies, various host animals can be immunized by injection with a peptide having a sequence that is present in Tango-63d and/or Tango-63e. Such host animals can include but are not limited to rabbits, mice, and rats, to name but a few. Various adjuvants can be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein (*Nature* 256:495-497, 1975;

and U.S. Patent No. 4,376,110), the human B cell hybridoma technique (Kosbor et al., *Immunology Today* 4:72, 1983; Cole et al., *Proc. Natl. Acad. Sci. USA* 80:2026-2030, 1983), and the EBV-hybridoma technique (Cole et al., "Monoclonal Antibodies And Cancer Therapy," Alan R. Liss, Inc., pp. 77-96, 1985). Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention can be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855, 1984; Neuberger et al., *Nature*, 312:604-608, 1984; Takeda et al., *Nature*, 314:452-454, 1985) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, *Science* 242:423-426, 1988; Huston et al., *Proc. Natl. Acad. Sci. USA* 85:5879-5883, 1988; and Ward et al., *Nature* 334:544-546, 1989) can be adapted to produce single chain antibodies against Tango-63d or Tango-63e gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes can be generated by known techniques. For example, such

fragments include but are not limited to: the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries can be constructed (Huse et al., *Science*, 246:1275-1281, 1989) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

These antibodies can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" Tango-63d or Tango-63e, using techniques well known to those skilled in the art. (See, for example, Greenspan and Bona, *FASEB J.* 7:437-444, 1993; and Nissinoff, *J. Immunol.* 147:2429-2438, 1991). Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in diagnostic regimens to detect disorders associated with apoptotic cell death.

Antibodies can be humanized by methods known in the art. For example, monoclonal antibodies with a desired binding specificity can be commercially humanized (Scotgene, Scotland; Oxford Molecular, Palo Alto, CA). Fully human antibodies, such as those expressed in transgenic animals are also features of the invention (Green et al., *Nature Genetics* 7:13-21, 1994; see also U.S. Patents 5,545,806 and 5,569,825, both of which are hereby incorporated by reference).

The methods described herein can be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one specific Tango-63d or Tango-63e nucleotide sequence or antibody reagent described herein, which can be conveniently used, for example, in clinical settings, to diagnose patients exhibiting symptoms of the disorders described below.

Animals, Intl. Rev. Cytol. 115:171-229. Skilled artisans can obtain additional guidance from, for example: Hogan et al. "Manipulating the Mouse Embryo" (Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1986; Krimpenfort et al., *Bio/Technology* 9:86, 1991; Palmiter et al., *Cell* 41:343, 1985; Kraemer et al., "Genetic Manipulation of the Early Mammalian Embryo" (Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1985; Hammer et al., *Nature* 315:680, 1985; Purcel et al., *Science*, 244:1281, 1986; Wagner et al., U.S. Patent 5,175,385; and Krimpenfort et al., U.S. Patent No. 5,175,384 (the latter two publications are hereby incorporated by reference).

The present invention provides for transgenic animals that carry the Tango-63-related transgene of the invention in all their cells, as well as animals which carry the transgene in some, but not all their cells, that is, the invention provides for mosaic animals. The transgene can be integrated as a single transgene or in concatamers, for example, head-to-head tandems or head-to-tail tandems. The transgene can also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (*Proc. Natl. Acad. Sci. USA* 89:6232-6236, 1992). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

When it is desired that the Tango-63d or Tango-63e transgene be integrated into the chromosomal site of the endogenous gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous Tango-63d or Tango-63e genes are designed for the purpose of integrating, via homologous recombination with chromosomal

sequences, into and disrupting the function of the nucleotide sequence of the endogenous Tango-63 gene. A transgene can also be selectively introduced into a particular cell type, thus inactivating or "knocking out" the endogenous gene in only that cell type, by following, for example, the teaching of Gu et al. (*Science* 265:103-106, 1994). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

The level of mRNA expression of the transgene in the tissues of the transgenic animals can be assessed using techniques which include but are not limited to Northern blot or RNAse protection analysis of tissue samples obtained from the animal.

Use of the Nucleic Acids, Polypeptides, and Antibodies of the Invention in the Diagnosis and Treatment of Disorders associated with Apoptotic Cell Death

As described herein, the nucleic acids, polypeptides, antibodies, and other reagents of the invention can be used in the diagnosis and treatment of disorders associated with apoptotic cell death. In general, disorders associated with decreased cell death are those in which the expression or activity of Tango-63d and/or Tango-63e can be insufficient. Thus, these disorders can be treated by enhancing the expression or activity of Tango-63d and/or Tango-63e. Conversely, disorders associated with increased cell death are those in which expression or activity of Tango-63d and/or Tango-63e is excessive, and which would respond to treatment regimes in which expression or activity of these genes is inhibited. The disorders amenable to treatment will first be briefly reviewed and a

discussion of therapeutic applications will follow (see, for example, "Formulations and Use").

In addition to the examples provided herein, skilled artisans can consult Thompson (*Science* 267:1456-1462, 1995) for additional discussion of the disorders associated with apoptotic cell death.

Whether a Disorder is Mediated by the Expression of Tango-63d or Tango-63e

If one can determine whether a disorder is associated with apoptotic cell death, and whether that cell death is influenced by expression of the polypeptides disclosed herein, it should be possible to determine whether that disorder can be diagnosed or treated with the nucleic acid, polypeptide, or antibody molecules of the invention. A disorder in which there is either insufficient or excessive cell death can be studied by determining whether Tango-63d or Tango-63e are either overexpressed or underexpressed in the affected tissue. The expression levels can be compared from tissue to tissue within a single patient, or between tissue samples obtained from a patient that is ill and one or more patients who are well. If it is determined that either Tango-63d, Tango-63e, or both are either overexpressed or underexpressed, it can be said that the disorder should be amenable to one or more of the treatment methods disclosed herein.

Diagnostic methods in which Tango-63d and Tango-63e are detected in a biological sample can be carried out, for example, by amplifying the nucleic acid molecules within the sample by PCR (the experimental embodiment set forth in Mullis, K.B., 1987, U.S. Patent No. 4,683,202), followed by the detection of the amplified molecules using techniques well known to those of skill in the art. For example, for

screening assays for therapeutic compounds on detection of Tango-63d polypeptide or Tango-63e polypeptide. Such assays for Tango-63d polypeptide or Tango-63e polypeptide, or peptide fragments thereof will typically involve incubating a sample, such as a biological fluid, a tissue extract, freshly harvested cells, or lysates of cells which have been incubated in cell culture, in the presence of a detectably labeled antibody capable of identifying these gene products (or peptide fragments thereof), and detecting the bound antibody by any of a number of techniques well-known in the art.

The biological sample can be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles, or soluble proteins. The support can then be washed with suitable buffers followed by treatment with the detectably labeled antibody or fusion protein. The solid phase support can then be washed with the buffer a second time to remove unbound antibody or fusion protein. The amount of bound label on solid support can then be detected by conventional means.

By "solid phase support or carrier" is intended any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material can have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration can be spherical, as in a bead, or cylindrical, as in the inside surface of a test

tube, or the external surface of a rod. Alternatively, the surface can be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of anti-Tango-63d or anti-Tango-63e antibody or fusion proteins containing these polypeptides can be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

With respect to antibodies, one of the ways in which the antibody of the instant invention can be detectably labeled is by linking it to an enzyme for use in an enzyme immunoassay (EIA) (Voller, A., "The Enzyme Linked Immunosorbent Assay (ELISA)", 1978, Diagnostic Horizons 2:1-7, Microbiological Associates Quarterly Publication, Walkersville, MD; Voller et al., *J. Clin. Pathol.* 31:507-520, 1978; Butler, *Meth. Enzymol.* 73:482-523, 1981; Maggio, E. (ed.), "Enzyme Immunoassay," CRC Press, Boca Raton, FL, 1980; Ishikawa, E. et al., (eds.), "Enzyme Immunoassay," Kgaku Shoin, Tokyo, 1981). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase,

glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection can also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection can also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect Tango-63d and Tango-63e through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, B., "Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques," The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to ~~label the~~ antibody with a ~~fluorescent compound~~. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound can be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in, which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Still further, the invention encompasses methods and compositions for the treatment of the disorders described above, and any others that are found to be associated with apoptotic cell death. Such methods and compositions are capable of modulating the level of expression of Tango-63d or Tango-63e and/or the level of activity of the gene products.

Numerous ways of altering the expression or activity of the polypeptides of the invention are known to skilled artisans. For example, living cells can be transfected *in vivo* with the nucleic acid molecules of the invention (or transfected *in vitro* and subsequently administered to the patient). For example, cells can be transfected with plasmid vectors by standard methods including, but not limited to, liposome- polybrene-, or DEAE dextran-mediated transfection (see, e.g., Felgner et al., *Proc. Natl. Acad.*

Sci. USA 84:7413, 1987; Ono et al., *Neurosci. Lett.* 117:259, 1990; Brigham et al., *Am. J. Med. Sci.* 298:278, 1989), electroporation (Neumann et al., *EMBO J.* 7:841, 1980), calcium phosphate precipitation (Graham et al., *Virology* 52:456, 1973; Wigler et al., *Cell* 14:725, 1978; Felgner et al., supra) microinjection (Wolff et al., *Science* 247:1465, 1990), or velocity driven microprojectiles ("biolistics").

These methods can be employed to mediate therapeutic application of the molecules of the invention. For example, antisense nucleic acid therapies or ribozyme approaches can be used to inhibit utilization of Tango-63d and/or Tango-63e mRNA; triple helix approaches can also be successful in inhibiting transcription of various polypeptides in the TNF receptor superfamily. Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to the mRNA molecules of the invention. The antisense oligonucleotides will bind to the complementary mRNA transcripts and prevent translation. Antisense oligonucleotides must be specific for the mRNA of interest. Accordingly, oligonucleotides disclosed herein as SEQ ID NOs:8, 9, 10, and 11 are especially preferred. For example, the following oligonucleotides are suitable for specifically binding Tango-63d or Tango-63e mRNA: 7

5'-CATGGCGGTAGGGAACGCTCT-3' (SEQ ID NO:8; the reverse and complement of nucleotides 128-148), 8

5'-GTTCTGTCCCCGTTGTTCCAT-3' (SEQ ID NO:9; the reverse and complement of nucleotides 110-130). The following oligonucleotides are suitable for specifically binding Tango-63d mRNA because they bind to sequences that are not present in Tango-63e: 5'-GGCTTCCCCACTGTGCTTTGT-3' (SEQ ID NO:10); and 5'-GGAGGTCACCGTCTCCTCCAC-3' (SEQ ID NO:11).

9 10

Absolute complementarity, although preferred, is not required. A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA can thus be tested, or triplex formation can be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it can contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation than oligonucleotides that are complementary to 5'- or 3'-untranslated sequence, but could be used in accordance with the instant invention. The antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects, the oligonucleotide is at least 10 nucleotides, preferably at least 17 nucleotides, more preferably at least 25 nucleotides, or most preferably at least 50 nucleotides.

Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies

compare levels of the target RNA or protein with that of an internal control RNA or protein. Additionally, it is envisioned that results obtained using an antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide can include other appended groups such as peptides (for example, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, for example, Letsinger et al., *Proc. Natl. Acad. Sci. USA* 86:6553-6556, 1989; Lemaitre et al., *Proc. Natl. Acad. Sci. USA* 84:648-652, 1987; PCT Publication No. WO88/09810, published December 15, 1988) or the blood-brain barrier (see, for example, PCT Publication No. WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents. (See, for example, Krol et al., *BioTechniques* 6:958-976, 1988) or intercalating agents (see, for example, Zon, *Pharm. Res.* 5:539-549, 1988). To this end, the oligonucleotide can be conjugated to another molecule, for example, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, and the like.

The antisense oligonucleotide can comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine,

oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., *Nucl. Acids Res.* 15:6131-6148, 1987), or a chimeric RNA-DNA analogue (Inoue et al., *FEBS Lett.* 215:327-330, 1987).

Oligonucleotides of the invention can be synthesized by standard methods known in the art, for example, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein et al. (*Nucl. Acids Res.* 16:3209, 1988), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., *Proc. Natl. Acad. Sci. USA* 85:7448-7451, 1988), etc.

The antisense molecules should be delivered to cells which express Tango-63d and/or Tango-63e *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA to cells; for example, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (for example, antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

However, it is often difficult to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs. Therefore a preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous Tango-63d and/or Tango-63e transcripts

and thereby prevent translation of the Tango-63d and/or Tango-63e mRNA. For example, a vector can be introduced *in vivo* such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, *Nature* 290:304-310, 1981), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., *Cell* 22:787-797, 1980), the herpes thymidine kinase promoter (Wagner et al., *Proc. Natl. Acad. Sci. USA* 78:1441-1445, 1981), the regulatory sequences of the metallothionein gene (Brinster et al., *Nature* 296:39-42, 1982), and so forth. Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct which can be introduced directly into the tissue site; for example, the choroid plexus or hypothalamus. Alternatively, viral vectors can be used which selectively infect the desired tissue; (for example, for brain, herpesvirus vectors can be used), in which case administration can be accomplished by another route (for example, systemically).

Methods of designing antisense nucleic acids and introducing them into host cells have been described in, for example, Weinberg et al. (U.S. Patent 4,740,463; hereby incorporated by reference).

Alternatively, the nucleic acid molecules of the invention can be administered so that expression of the Tango-63d and/or Tango-63e occurs in tissues where it does not normally occur, or is enhanced in tissues where it is normally expressed. This application can be used, for example, to suppress apoptotic cell death and thereby treat disorders in which cellular populations are diminished, such as those described herein as "disorders associated with diminished cell survival." Preferably, the therapeutic nucleic acid (or recombinant nucleic acid construct) is applied to the site where cells are at risk of dying by apoptosis, to the tissue in the larger vicinity, or to the blood vessels supplying these areas.

Ideally, the production of a polypeptide that is a form of Tango-63d or Tango-63e (including forms that are involved in mediating apoptosis) by any gene therapy approach described herein, will result in a cellular level of expression that is at least equivalent to the normal, cellular level of expression of Tango-63d or Tango-63e. Skilled artisans will recognize that these therapies can be used in combination with more traditional therapies, such as surgery, radiotherapy, or chemotherapy. Accordingly, and as described below, the invention features therapeutic compositions that contain the nucleic acid molecules, polypeptides, and antibodies of the invention, as well as compounds that are discovered, as described below, to affect them.

Therapeutic Compositions

The nucleic acid molecules encoding Tango-63d and Tango-63e, the polypeptides themselves, antibodies that specifically bind Tango-63d and/or Tango-63e and compounds that affect the expression or activity of Tango-63d or Tango-63e can be administered to a patient at therapeutically effective doses to treat or ameliorate disorders associated with apoptotic cell death. A therapeutically effective dose refers to the dose that is sufficient to result in amelioration of symptoms of disorders associated with apoptotic cell death.

Effective Dose

Toxicity and therapeutic efficacy of a given compound can be determined by standard pharmaceutical procedures, using either cells in culture or experimental animals to determine the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to unaffected cells and, thereby, reduce the danger or severe side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage can vary within this range depending upon the dosage form

compound and a suitable powder base such as lactose or starch.

The compounds can be formulated for parenteral administration by injection, for example, by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, for example, in ampoules or in multi-dose containers, with an added preservative. The compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient can be in powder form for constitution with a suitable vehicle, for example, sterile pyrogen-free water, before use.

The compounds can also be formulated in rectal compositions such as suppositories or retention enemas, for example, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds can also be formulated as a depot preparation. Such long acting formulations can be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds can be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions can, if desired, be presented in a pack or dispenser device which can contain one or more unit dosage forms containing the active ingredient. The pack can for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device can be accompanied by instructions for administration.

The therapeutic compositions of the invention can also contain a carrier or excipient, many of which are known to skilled artisans. Excipients which can be used include buffers (for example, citrate buffer, phosphate buffer, acetate buffer, and bicarbonate buffer), amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins (for example, serum albumin), EDTA, sodium chloride, liposomes, mannitol, sorbitol, and glycerol. The nucleic acids, polypeptides, antibodies, or modulatory compounds of the invention can be administered by any standard route of administration. For example, administration can be parenteral, (for example, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, opthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, or transmucosal administration) or oral. The modulatory compound can be formulated in various ways, according to the corresponding route of administration. For example, liquid solutions can be made for ingestion or injection; gels or powders can be made for ingestion, inhalation, or topical application. Methods for making such formulations are well known and can be found in, for example, "Remington's Pharmaceutical Sciences." It is expected that the preferred route of administration will be intravenous.

It is well known in the medical arts that dosages for any one patient depend on many factors, including the general health, sex, weight, body surface area, and age of the patient, as well as the particular compound to be administered, the time and route of administration, and other drugs being administered concurrently.

Dosages for the polypeptides and antibodies of the invention will vary, but a preferred dosage for intravenous administration is approximately 0.01 mg to 100 mg/ml blood

volume. Determination of the correct dosage within a given therapeutic regime is well within the abilities of one of ordinary skill in the art of pharmacology. Skilled artisans will be aided in their determination of an adequate dosage by previous studies. For example, Abraham et al. (*J. Amer. Med. Assoc.* 273:934-941, 1995) administered TNF-α monoclonal antibody (TNF-α-MAb) at doses ranging from 1 to 15 mg/kg. The antibody was well tolerated by all patients, even though they developed human antimurine antibodies; no serum sickness-like reactions, adverse skin reactions, or systemic allergic reactions developed. Similarly, Rankin et al. (*Br. J. Rheumatol.* 34:334-342, 1995) administered a single intravenous dose of 0.1, 1.0, or 10 mg/kg of an engineered human antibody, CDP571, which neutralizes human TNF-α. Both studies describe in detail how to evaluate patients who have been treated with antibodies.

Identification of Compounds that mediate Oligomerization between Polypeptides within a Tango-63d- or Tango-63e-containing Complex

It has been shown (see Background of the Invention) that apoptosis can be induced by the formation of specific complexes of polypeptides, for example those that assemble when TNFR-1 or the Fas receptor are bound. Given the conservation between the intracellular domains of TNFR-1, Tango-63d, and Tango-63e, the same or similar polypeptides may assemble with Tango-63d or Tango-63e. Therefore, apoptosis can be inhibited within a cell that contains compounds that specifically inhibit interaction between Tango-63d and/or Tango-63e and polypeptides that would otherwise assemble to form a complex with these polypeptides. ~~Conversely, apoptosis can be stimulated within a cell containing compounds that specifically promote~~

interaction between Tango-63d and/or Tango-63e and one or more additional polypeptides. Accordingly, the invention features a method for treating a patient who has a disorder associated with an abnormally high rate of apoptotic cell death by administering to the patient a compound that inhibits oligomerization between Tango-63d or Tango-63e and other polypeptides. Patients who suffer instead from an abnormally low rate of apoptotic cell death can be treated with a compound that promotes oligomerization between these polypeptides.

The invention also features methods for screening compounds to identify those which increase or decrease the interaction between either Tango-63d and Tango-63e and other polypeptides. One suitable assay for determining whether another polypeptide has become associated with Tango-63d or Tango-63e is an immuprecipitation assay. A suitable immunoprecipitation assay is described by Kischkel et al. (*EMBO J.* 14:5579, 1995). Anti-Tango-63d or Anti-Tango-63e antibodies can be used to perform these assays in the presence and absence of selected compounds, and to thereby identify those that increase or decrease association between polypeptides within the Tango-63d and Tango-63e complexes.

Recently, compounds that can penetrate the cell membrane were devised and shown to be capable of controlling the intracellular oligomerization of specific proteins. More specifically, ligands were used to induce intracellular oligomerization of cell surface receptors that lacked their transmembrane and extracellular regions but that contained intracellular signaling domains. Spencer et al. (*Science* 262:1019-1024, 1993) reported that addition of these ligands to cells in culture resulted in signal transmission and specific target gene activation. Further, these investigators proposed the use of these ligands "wherever

precise control of a signal transduction pathway is desired." For further guidance in the use of synthetic ligands to induce dimerization of proteins, see Belshaw et al. (*Proc. Natl. Acad. Sci. USA* 93:4604-4607). This approach can be used to induce intracellular oligomerization within a Tango-63d- or Tango-63e-containing complex.

Identification of Compounds that Modulate the Expression or Activity of Tango-63d or Tango-63e

Isolation of the nucleic acid molecules described above (i.e. those encoding Tango-63d and Tango-63e) also facilitates the identification of compounds that can increase or decrease the expression of these molecules *in vivo*. To discover such compounds, cells that express Tango-63d and/or Tango-63e are cultured, exposed to a test compound (or a mixture of test compounds), and the level of Tango-63d and/or Tango-63e expression or activity is compared with the level of expression or activity in cells that are otherwise identical but that have not been exposed to the test compound(s). Many standard quantitative assays of gene expression can be utilized in this aspect of the invention. Examples of these assays are provided below.

In order to identify compounds that modulate expression of Tango-63d or Tango-63e (or homologous genes), the candidate compound(s) can be added at varying concentrations to the culture medium of cells that express Tango-63d or Tango-63e, as described above. These compounds can include small molecules, polypeptides, and nucleic acids. The expression of Tango-63d and Tango-63e is then measured, for example, by Northern blot, PCR analyses or RNAse protection analyses using a nucleic acid molecule of the invention as a probe. The level of expression of the polypeptides of the invention in the presence of the

Human prostate epithelial cells were obtained from Clonetics Corporation (San Diego, CA) and expanded in culture with Prostate Epithelial Growth Medium (PrEGM; Clonetics) according to the recommendations of the supplier. When the cells reached 80% confluence, they were cultured in Prostate Basal Media (Clonetics) for 24 hours. The prostate cells were then stimulated with PrEGM and cycloheximide (CHI; 40 μg/ml) for 3 hours. Total RNA was isolated using the RNeasy™ Midi Kit (Qiagen; Chatsworth, CA), and the polyA+ fraction was further purified using Oligotex™ beads (Qiagen).

Three μg of polyA+ RNA were used to synthesize a cDNA library using the Superscript™ cDNA synthesis kit (Gibco BRL, Gaithersburg, MD). Complementary DNA was directionally cloned into the expression plasmid pMET7 using the *SalI* and *NotI* sites in the polylinker to construct a plasmid library. Transformants were picked and grown up for single-pass sequencing. Additionally, prostate cDNA was ligated into the *SalI/NotI* sites of the ZipLox™ vector (Gibco BRL) for construction of a lambda phage cDNA library.

Two different forms of Tango-63 have been identified in the prostate cDNA library through EST sequencing and screening of the lambda phage library for the isolation of additional clones (Tango-63d and Tango-63e). Tango-63d encodes a polypeptide of 440 amino acids (encoded by nucleotides 128 to 1447 of SEQ ID NO: 1 and shown in Fig. 1); and Tango-63e encodes a polypeptide of 411 amino acids (encoded by nucleotides 128 to 1360 of SEQ ID NO: 3 nad shown in Fig. 2). The polypeptide encoded by Tango-63e is identical to that encoded by Tango-63d, with the exception of the deletion of amino acids 183-211 (encoded by nucleotides 677-760) in the Tango-63d sequence. The deleted amino acids are those just amino-terminal to the

transmembrane domain in Tango-63d. Tango-63d and Tango-63e are novel polypeptides that represent new members of the tumor necrosis factor (TNF) receptor superfamily.

The members of the TNFR receptor superfamily are characterized by the presence of cysteine-rich repeats in their extracellular domains, and the Fas/APO-1 receptor and TNFR-1 also share an intracellular region of homology designated the "death domain" because it is required to signal apoptosis (Itoh and Nagata, *J. Biol. Chem.* 268:10932-10937, 1993). As described above, this shared death domain suggests that both receptors interact with a related set of signal-transducing molecules.

Tissue Distribution of Tango-63

The expression of Tango-63 (which is subsequently alternatively spliced to produce the novel polypeptides of the invention, Tango-63d and Tango-63e) was analyzed using Northern bloc hybridization. A 422 base pair DNA fragment was generated using PCR with the following two oligonucleotides: LRH1 (5'-ATGGAACAACGGGGACAG-3' (SEQ ID NO:6); nucleotide positions 128-145 in Tango-63d) and LRH3 (5'-TTCTTCGCACTGACACAC-3' (SEQ ID NO:7); reverse and complement to nucleotide positions 533-550 in Tango-63d for use as a probe. The DNA was radioactively labeled with ^{32}P-dCTP using the Prime-It™ kit (Stratagene, La Jolla, CA) according to the instructions of the supplier. Filters containing human mRNA (MTNI and MTNII from Clontech, Palo Alto, CA) were probed in ExpressHyb™ hybridization solution (Clontech) and washed at high stringency. More specifically, the wash was carried out by submerging the filters in 2X SSC, 0.05% SDS at 55°C (2 X 20 minutes) and then in 0.1X SSC, 0.1% SDS at 55°C (2 X 20 minutes).

Tango-63 is expressed as a 4.2 kilobase (kb) transcript in a wide variety of human tissues including heart, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovaries, small intestine, colon, and peripheral blood leukocytes. Expression of Tango-63 was also detectable in the brain, but at significantly lower levels than in other tissues. Additional, but fainter, bands at about 2.2 kb (liver) and 1.0 kb (skeletal muscle) were also observed. These bands could represent additional forms of Tango-63, degradation products, or cross-reacting mRNAs.

An Assay for Tango-63d and Tango-63e Mediated Apoptosis

An assay for Tango-63d- or Tango-63e-mediated apoptosis can be used in screening assays to identify compounds that increase or decrease the degree of apoptosis within a population of cells. The compounds identified using these assays can alter the degree of apoptosis by altering the expression of Tango-63d or Tango-63e, the activity of Tango-63d or Tango-63e, or the way in which these polypeptides interact with other polypeptides. Compounds identified in these assays can be used as therapeutic compounds to treat disorders associated with an abnormal rate of apoptosis.

Assays of apoptosis, particularly when apoptosis is mediated by a polypeptide in the TNF receptor superfamily, generally employ an antibody directed against the polypeptide, which, upon binding, initiates apoptosis. Alternatively, an assay that requires only overexpression of the polypeptide of interest can be performed. An example of such an assay is described below.

The activity of the polypeptides of the invention can be assayed via a cotransfection assay that is based on co-uptake (transfection) with plasmids that encode a polypeptide of the invention. The assay described below is based on the observation that overexpression of TNFR-1, DR-3, and several other death inducing molecules, such as Caspases, is sufficient to cause apoptosis in the absence of other stimuli. The assay described below demonstrates the ability of the novel polypeptides of the invention to diminish the number of cells surviving in culture by activating apoptosis.

β-galactosidase expression assays were performed essentially as described by Kumar et al. (*Genes & Dev.* 8:1613-1626, 1994). SW480 cells, derived from a human colon carcinoma, were cultured in Dulbecco's modified Eagle's medium (DMEM), high glucose, supplemented with 10% fetal calf serum and 100 μg/ml each of penicillin G and streptomycin. The cells were seeded at a density of 3 X 10^5 cells/well on 6-well (35 mm) plates and grown in 5% CO_2 at 37°C. The following day, the cells were transfected with 0.5 μg of pSVβ (Clontech), which carries an insert encoding β-galactosidase, and 2.5 μg of either a control or an experimental plasmid using Lipofectamine™ reagent (Life Technologies) and Opti-MEM™ medium (Life Technologies). The experimental plasmids contained inserts encoding Tango-63d or Tango-63e; the control plasmids were otherwise identical except the Tango-63d or Tango-63e inserts were absent. Thirty-six hours following transfection, the cells were rinsed twice with phosphate-buffered saline (PBS), fixed, and stained for 6 hours or more at 37°C. If desired, the cells can remain in the staining solution at room temperature for longer periods of time. The staining process consisted of exposure to 1% X-gal, 4 mM potassium

ferricyanide, and 2 mM magnesium chloride in PBS. After staining, the cells were examined with a light microscope for the appearance of blue color, indicating successful transfection.

The result of transfection with the control plasmid (encoding β-gal) and either the control or experimental plasmid (encoding Tango-63d or Tango-63e) was assessed by determining the percentage of blue (i.e. transfected) cells in each well or by counting the total number of blue cells in each well. In preliminary experiments, expression of Tango-63d or Tango-63e caused approximately 90% reduction in the number of β-gal positive cells remaining in culture.

Numerous substances are capable of inducing apoptosis in various cell types and can thus be used in assays of apoptosis. These substances include physiological activators, such as TNF family members (for example, Fas ligand, TNFα, and TRAIL/APO2). Cell death can also be induced when growth factors are withdrawn from the medium in which the cells are cultured. Additional inducers of apoptosis include heat shock, viral infection, bacterial toxins, expression of the oncogenes *myc*, *rel*, and *E1A*, expression of tumor suppressor genes, cytolytic T cells, oxidants, free radicals, gamma and ultraviolet irradiation, β-amyloid peptide, ethanol, and chemotherapeutic agents such as Cisplatin, doxorubicin, arabinoside, nitrogen mustard, methotrexate, and vincristine.

Example 3

Expression of Recombinant Tango-67 in COS cells

A vector for expression of Tango-67 can be prepared using a vector pcDNAI/Amp (Invitrogen). This vector includes: a SV40 origin of replication, an ampicillin resistance gene, an E. coli replication origin, a CMV promoter followed by polylinker region, a SV40 intron, a and polyadenylation site. A DNA fragment encoding Tango-67 is cloned into the polylinker region of the vector such that Tango-67 expression is under the control of the CMV promoter. A DNA sequence encoding Tango-67 is prepared by PCR amplification of a Tango-67 using primers which include restriction sites that are compatible with the polylinker. The Tango-67 sequence is inserted into the vector. The resulting construct is used to transform E. coli strain SURE (Stratagene, La Jolla, CA) and amp resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis the presence of the correct fragment. For expression of the recombinant Tango-67, COS cells are transfected with the expression vector by DEAE-DEXTRAN method and grown is standard tissue culture medium.

Chromosome 8p Loss of Heterozygosity (LOH) and Tango-63

In tumor tissues and cultured cancer cells, loss of heterozygosity (LOH) is much more frequently observed on the short arm of human chromosome 8p than on any other human chromosome. Tumor suppressor genes have been identified in regions of frequent LOH in tumor samples (e.g., p53, Rb, APC, DCC-DPC4). The frequency of LOH reported in the 8p region defined by markers D8S133 to NEFL is greater than 80% in prostate cancer microdissected samples (Vocke et al., *Cancer Res.* 56:2411-2416, 1996). In addition, loss of 8p is

also a frequent event in a number of other cancers including colon cancer, non-small cell lung cancer, breast cancer (Yaremko et al., *Genes, Chrom. Cancer* 16:189-195, 1996), head and neck cancer (Scholnick et al., *J. Natl. Cancer Inst.* 88:1676-1682, 1996), hepatocarcinoma (Emi et al., *Genes, Chrom. Cancer* 7:152-157, 1993), and bladder cancer (Takle et al., *Oncogene* 12:1083-1087, 1996). Linkage analyses on German breast cancer families' pedigrees have identified a strong linkage in this same region of 8p (Seitz et al., *Oncogene* 14:741-743, 1997), which has been termed the BRCA3 gene region (Kerangueven et al.).

Tango-63 has been mapped on the Stanford Human Genome Center G3 radiation hybrid panel close to marker D8S1734 with a LOD score of 6. The mapping was carried out using a pair of primers from the 3' untranslated region (UTR). The primers are designated t63-f2 (5'-ATGTCATTGTTTTCACAGCA-3'; SEQ ID NO:12) and t63-r2 (5'-GCTCAAGCGATTCTCTCA-3'; SEQ ID NO:13). This map position is located in the most frequently lost region of chromosome 8 between markers D8S133 and NEFL.

Subsequently, three overlapping yeast artificial chromosomes (YACs) were used to place Tango-63 on the physical map of chromosome 8 between markers WI-6088 and WI-6563.

Deposit Information

Two plasmids bearing cDNA encoding Tango-63d and Tango-63e respectively, were deposited with the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852-1776) on February 13, 1997. The plasmid encoding Tango-63d was assigned accession number 98368, and the plasmid encoding Tango-63e was assigned accession number 98367.

The subject cultures have been deposited under conditions that assure that access to the cultures will be available during the pendency of the patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject culture deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., they will be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposits, and in any case, for a period of at least 30 (thirty) years after the date of deposit or for the enforceable life of any patent which can issue disclosing the cultures plus five years after the last request for a sample from the deposit. The depositor acknowledges the duty to replace the deposits should the depository be unable to furnish a sample when requested, due to the condition of the deposits. All restrictions on the availability to the public of the subject culture deposits will be irrevocably removed upon the granting of a patent disclosing them.

Additional embodiments are within the following claims.

What is claimed is:

1. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide that is at least 85% identical to SEQ ID NO:2.

2. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide that is at least 85% identical to SEQ ID NO:4.

3. The nucleic acid molecule of claim 1 or claim 2, said molecule encoding a polypeptide that associates with the cell surface and mediates the cellular response to an apoptotic signal.

4. The nucleic acid molecule of claim 1, said molecule encoding the amino acid sequence of SEQ ID NO:2.

5. The nucleic acid molecule of claim 4, said molecule comprising the nucleotide sequence of SEQ ID NO:1.

6. The nucleic acid molecule of claim 2, said molecule encoding the amino acid sequence of SEQ ID NO:4.

7. The nucleic acid molecule of claim 6, said molecule comprising the nucleotide sequence of SEQ ID NO:3.

8. An isolated nucleic acid molecule, said molecule comprising the cDNA sequence contained within ATCC Accession No. 98367.

9. An isolated nucleic acid molecule, said molecule comprising the cDNA sequence contained within ATCC Accession No. 98368.

10. A vector comprising the nucleic acid molecule of claim 1, claim 2, claim 4, or claim 6.

11. The vector of claim 10, said vector being an expression vector.

12. The vector of claim 11, further comprising a regulatory element.

13. The vector of claim 12, wherein the regulatory element is selected from the group consisting of the cytomegalovirus hCMV immediate early gene, the early promoter of SV40 adenovirus, the late promoter of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage λ, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase, the promoters of acid phosphatase, and the promoters of the yeast α-mating factors.

14. The vector of claim 12, wherein said regulatory element directs tissue-specific expression.

15. The vector of claim 10, further comprising a reporter gene.

16. The vector of claim 15, wherein the reporter gene is selected from the group consisting of β-lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo^r, $G418^r$), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase

(TK), lacZ (encoding β-galactosidase), and xanthine guanine phosphoribosyltransferase (XGPRT).

17. The vector of claim 10, wherein said vector is a plasmid.

18. The vector of claim 10, wherein said vector is a virus.

19. The vector of claim 18, wherein said virus is a retrovirus.

20. A genetically engineered host cell comprising the expression vector of claim 11.

21. The cell of claim 20, wherein said cell is eukaryotic.

22. A substantially pure polypeptide having the amino acid sequence encoded by the nucleic acid molecule of claim 1, claim 2, claim 4, or claim 6.

23. The polypeptide of claim 22, further comprising a heterologous polypeptide other than a Caspase-8 polypeptide.

24. An antibody that specifically binds Tango-63d or Tango-63e.

25. The antibody of claim 24, wherein said antibody is a neutralizing antibody.

26. A transgenic animal harboring the nucleic acid molecule of claim 1, claim 2, claim 4, or claim 6.

27. A method of determining whether a patient has a disorder associated with an abnormal rate of apoptotic cell death, said method comprising quantitating the level of Tango-63d expression in a biological sample obtained from said patient.

28. The method of claim 27, comprising quantitating mRNA encoding Tango-63d.

29. The method of claim 27, comprising quantitating Tango-63d protein.

30. A method of determining whether a patient has a disorder associated with an abnormal rate of apoptotic cell death, said method comprising quantitating the level of Tango-63e expression in a biological sample obtained from said patient.

31. The method of claim 30, comprising quantitating mRNA encoding Tango-63e.

32. The method of claim 30, comprising quantitating Tango-63e protein.

33. The method of claim 28 or claim 31, comprising an RNAse protection assay, Northern blot analysis, or amplification by RT-PCR.

34. The method of claim 29 or claim 32, comprising Western blot analysis.

35. The method of claim 27 or claim 30, wherein said biological sample is a tumor sample.

36. A method of treating a patient who has a disorder associated with abnormal expression or activity of Tango-63d, said method comprising administering to the patient a compound that modulates the expression or activity of Tango-63d.

37. The method of claim 36, wherein the compound comprises a small molecule, an antisense nucleic acid molecule, or a ribozyme.

38. A method of treating a patient who has a disorder associated with abnormal expression or activity of Tango-63e, said method comprising administering to the patient a compound that modulates the expression or activity of Tango-63e.

39. The method of claim 38, wherein the compound comprises a small molecule, an antisense nucleic acid molecule, or a ribozyme.

40. A therapeutic composition comprising the compound of claim 36 or claim 38.

41. A method for treating a patient who has a disorder associated with abnormal activity of the Tango-63d receptor complex, said method comprising administering a compound that mediates oligomerization between Tango-63d and one or more of the polypeptides that form a Tango-63d receptor complex.

42. A method for treating a patient who has a disorder associated with abnormal activity of the Tango-63e receptor complex, said method comprising administering a compound that modulates activity of said complex.

43. The method of claim 42, wherein said compound mediates oligomerization between Tango-63e and one or more of the polypeptides that form a Tango-63e receptor complex.

44. A method for treating a patient who has a disorder associated with abnormal expression of Tango-63e or a member of the Tango-63e receptor complex, said method comprising administering a compound that modulates expression of Tango-63e or a member of the Tango-63e complex.

45. The method of claim 41 or claim 42, wherein the patient has a disorder in which the rate of apoptotic cell death is abnormally low.

46. The method of claim 41 or claim 42, wherein the compound is synthetic.

47. A method of treating a patient who has a disorder associated with excessive apoptotic cell death, said method comprising administering to the patient the nucleic acid molecule of claim 1 or claim 2, wherein said molecule encodes a dysfunctional polypeptide.

48. A method of treating a patient who has a disorder associated with excessive apoptotic cell death, said method comprising administering to the patient the

polypeptide of claim 22, wherein said polypeptide is dysfunctional.

49. A method of identifying a compound that modulates expression of Tango-63d, said method comprising assessing the expression of Tango-63d in the presence and absence of said compound.

50. A method of identifying a compound that modulates expression of Tango-63e, said method comprising assessing the expression of Tango-63e in the presence and absence of said compound.

51. A method for treating a patient who has a disease characterized by an abnormally low rate of apoptotic cell death, said method comprising administering a compound that mediates oligomerization between Tango-63d and one or more of the polypeptides that form a Tango-63d receptor complex.

52. A method for treating a patient who has a disease characterized by an abnormally low rate of apoptotic cell death, said method comprising administering a compound that mediates oligomerization between Tango-63e and one or more of the polypeptides that form a Tango-63e receptor complex.

53. A method of identifying a compound that modulates the activity of Tango-63d, said method comprising assessing the activity of Tango-63d in the presence and absence of said compound.

54. A method of identifying a compound that modulates the activity of Tango-63e, said method comprising assessing the activity of Tango-63e in the presence and absence of said compound.

55. A method for determining whether a selected compound modulates oligomerization between Tango-63d and one or more of the polypeptides that form a Tango-63d receptor complex, said method comprising measuring oligomerization of the Fas/APO-1 receptor complex and Tango-63d and one or more of the polypeptides that form a Tango-63d receptor complex in the presence and absence of said selected compound.

56. A method for determining whether a selected compound modulates oligomerization between Tango-63d and one or more of the polypeptides that form a Tango-63e receptor complex, said method comprising measuring oligomerization of Tango-63e and one or more of the polypeptides that from a Tango-63e receptor complex in the presence and absence of said selected compound.

57. An isolated nucleic acid molecule which hybridizes under stringent conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, said isolated nucleic acid molecule encoding Tango-63d.

58. An isolated nucleic acid molecule which hybridizes under stringent conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:3, said isolated nucleic acid molecule encoding Tango-63e.

59. An isolated nucleic acid molecule comprising a nucleotide sequence which is at least 90% identical to the nucleotide sequence of SEQ ID NO:1, said isolated nucleic acid molecule encoding Tango-63d.

60. An isolated nucleic acid molecule comprising a nucleotide sequence which is at least 90% identical to the nucleotide sequence of SEQ ID NO:3, said isolated nucleic acid molecule encoding Tango-63e.

61. The method of claim 47, wherein said dysfunctional polypeptide comprises a mutation that inhibits ligand binding.

62. The method of claim 47, wherein said dysfunctional polypeptide comprises a mutation that inhibits formation of a receptor complex.

63. A method of identifying a ligand capable of binding a polypeptide having an amino acid sequence encoded by the nucleic acid molecule of claim 1, claim 2, claim 4, or claim 6, said method comprising contacting said polypeptide with said ligand, and determining whether a complex forms between said ligand and said polypeptide.

64. An isolated nucleic acid molecule that hybridizes under stringent conditions to cDNA sequence contained within ATCC Accession No. 98367.

65. An isolated nucleic acid molecule that hybridizes under stringent conditions to cDNA sequence contained within ATCC Accession No. 98368.

66. An isolated nucleic acid molecule that is 85% identical to SEQ ID NO:1 (Fig. 1).

67. An isolated nucleic acid molecule that is 85% identical to SEQ ID NO:3 (Fig. 2).

68. An isolated nucleic acid molecule that is 95% identical to SEQ ID NO:1.

69. An isolated nucleic acid molecule that is 95% identical to SEQ ID NO:3.

70. An isolated nucleic acid molecule that is 85% identical to cDNA sequence contained within ATCC Accession No. 98367.

71. An isolated nucleic acid molecule that is 85% identical to cDNA sequence contained within ATCC Accession No. 98368.

72. An isolated nucleic acid molecule that is 95% identical to cDNA sequence contained within ATCC Accession No. 98367.

73. An isolated nucleic acid molecule that is 95% identical to cDNA sequence contained within ATCC Accession No. 98368.

74. An isolated nucleic acid molecule that hybridizes under stringent conditions to nucleotides 128 to 1447 of SEQ ID NO:1 (Fig. 1).

75. An isolated nucleic acid molecule that hybridizes under stringent conditions to nucleotides 128 to 1360 of SEQ ID NO:3 (Fig. 2).

76. The polypeptide encoded by the nucleic acid molecule of claim 64.

77. The polypeptide encoded by the nucleic acid molecule of claim 65.

78. The polypeptide encoded by the nucleic acid molecule of claim 66.

79. The polypeptide encoded by the nucleic acid molecule of claim 67.

80. The polypeptide encoded by the nucleic acid molecule of claim 68.

81. The polypeptide encoded by the nucleic acid molecule of claim 69.

NOVEL POLYPEPTIDES WITHIN THE TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY AND USES THEREFOR

Abstract of the Disclosure

The present invention relates to the discovery and characterization of two novel polypeptides within the tumor necrosis factor (TNF) receptor superfamily. The first receptor of the invention, Tango-63d, encodes a 440 amino acid polypeptide and the second receptor of the invention, Tango-63e, encodes a 411 amino acid polypeptide.

The invention encompasses nucleic acid molecules encoding Tango-63d and Tango-63e or mutant forms thereof which encode dysfunctional receptor polypeptides, vectors containing these nucleic acid molecules, cells harboring recombinant DNA molecules encoding Tango-63d and/or Tango-63e or mutant forms thereof, host fusion proteins which include functional or dysfunctional Tango-63d or Tango-63e, transgenic animals which express Tango-63d and/or Tango-63e, screening methods and therapeutic methods employing the nucleic acid molecules and polypeptides described above, substantially purified Tango-63d, substantially purified Tango-63e and therapeutic compositions containing these nucleic acid molecules and polypeptides.

234854.B11

Attorney's Docket No.: 07334-

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled NOVEL POLYPEPTIDES WITHIN THE TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY AND USES THEREFOR, the specification of which:

[] is attached hereto.

[X] was filed on April 16, 1997 as Application Serial No. 08/843,652 and was amended on _____

[] was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance w Title 37, Code of Federal Regulations, §1.56.

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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M E Q R G Q N 7 SEQ ID NO
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FIG. 1 (sheet 1 of 3)

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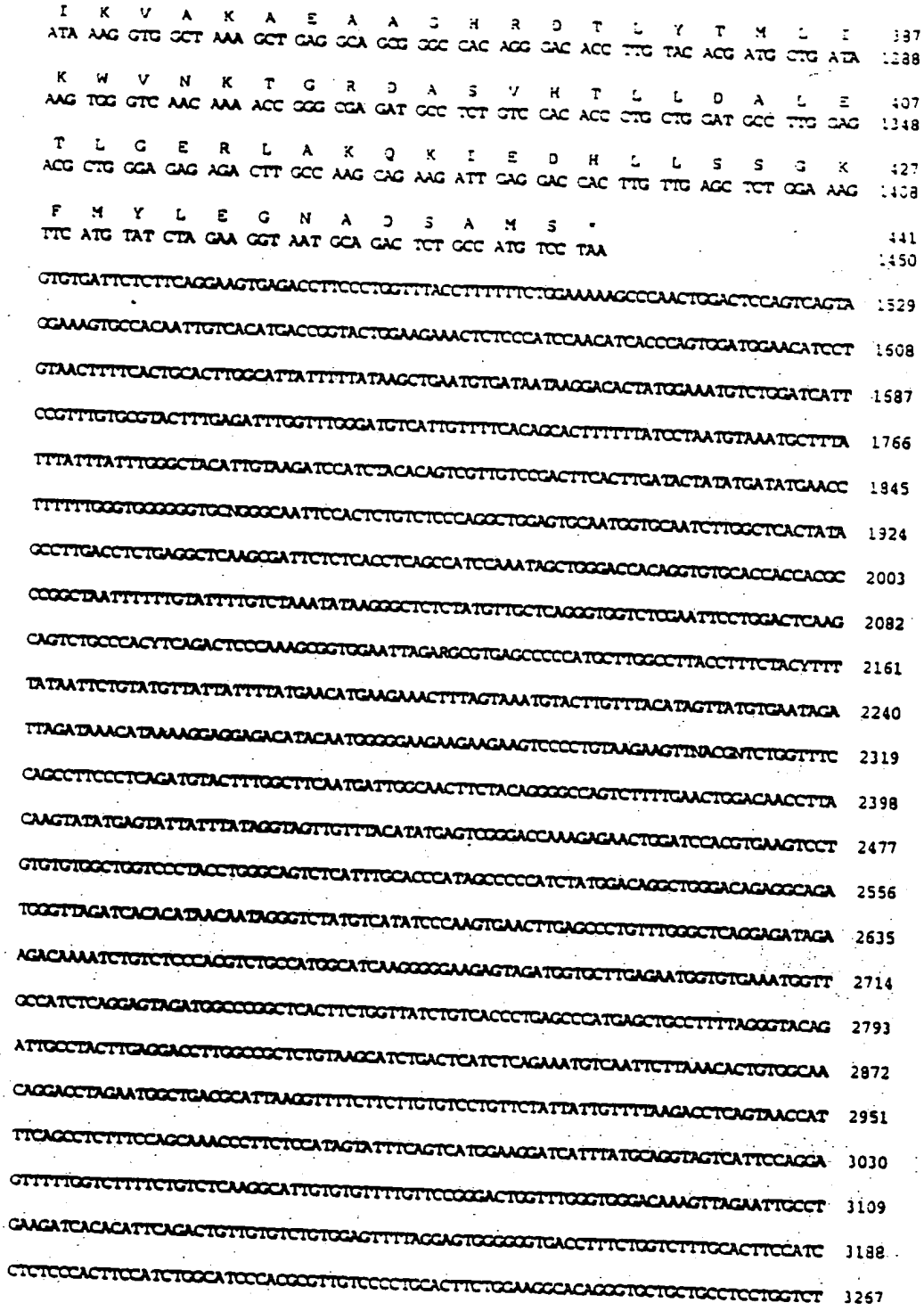

FIG. 1 (sheet 2 of 3)

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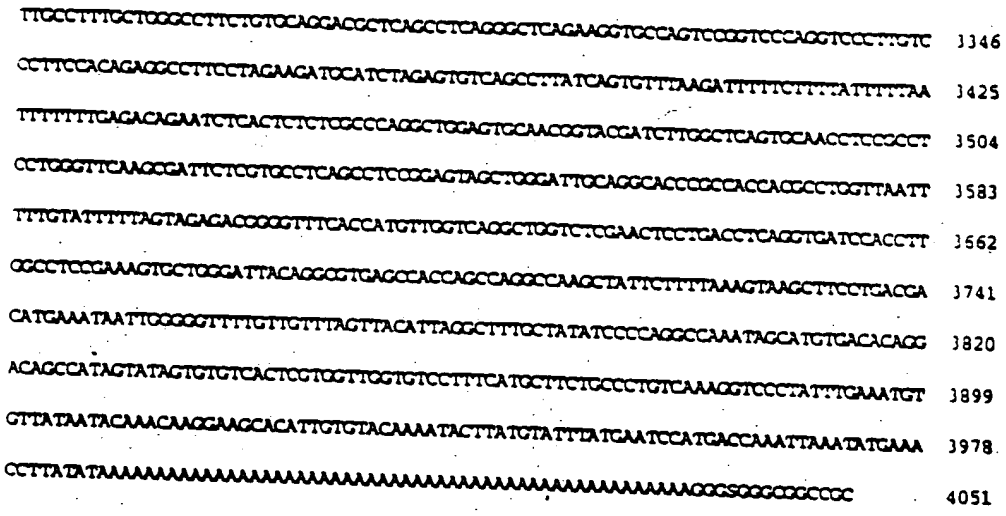

FIG. 1 (sheet 3 of 3)

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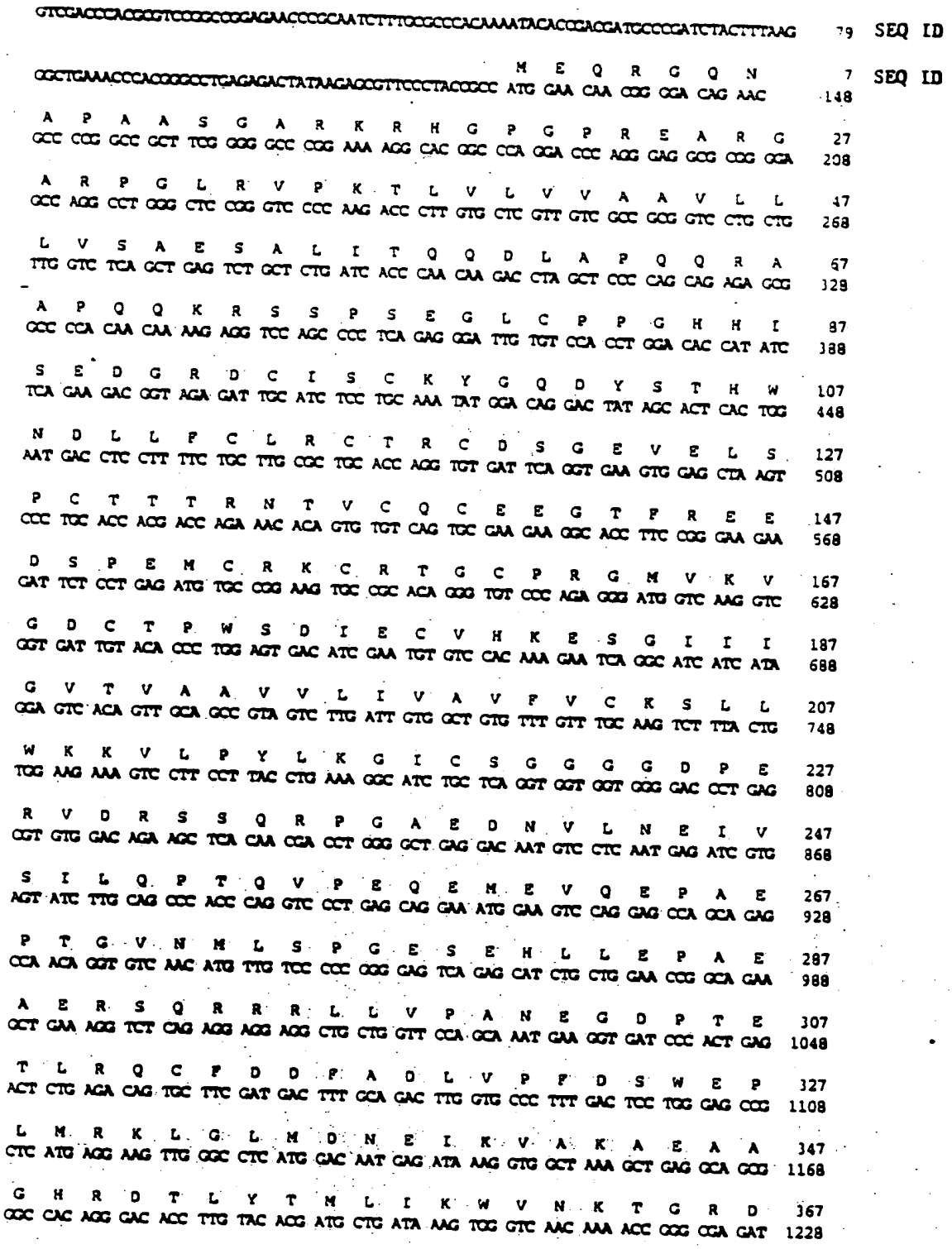

FIG. 2 (sheet 1 of 3)

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 K  I  E  D  H  L  L  S  S  G  K  F  M  Y  L  E  G  N  A  D   407
AAG ATT GAG GAC CAC TTG TTG AGC TCT GGA AAG TTC ATG TAT CTA GAA GGT AAT GCA GAC  1348

 S  A  M  S  *                                                412
TCT GCC ATG TCC TAA                                           1363

GTGTGATTCTCTTCAGGAAGTGAGACCTTCCCTGGTTTACCTTTTTTCTGGAAAAGCCCAACTGGACTCCAGTCAGTA  1442

GGAAAGTGCCACAATTGTCACATGACCGGTACTGGAAGAAACTCTCCCATCCAACATCACCCAGTGGATGGAACATCCT  1521

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CAGCCTTCCCTCAGATGTACTTTGGCTTCAATGATTGGCAACTTCTACAGGGGCCAGTCTTTTGAACTGGACAACCTTA  2311

CAAGTATATGAGTATTATTTATAGGTAGTTGTTTACATATGAGTCGGGACCAAAGAGAACTGGATCCACGTGAAGTCCT  2390

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AGACAAAATCTGTCTCCCACGTCTGCCATGGCATCAAGGGGGAAGAGTAGATGGTGCTTGAGAATGGTGTGAAATGGTT  2627

GCCATCTCAGGAGTAGATGGCCCCGCTCACTTCTGGTTATCTGTCACCCTGAGCCCATGAGCTGCCTTTTAGGGTACAG  2706

ATTGCCTACTTGAGGACCTTGGCCGCTCTGTAAGCATCTGACTCATCTCAGAAATGTCAATTCTTAAACACTGTGGCAA  2785

CAGGACCTAGAATGGCTGACGCATTAAGGTTTTCTTCTTGTGTCCTGTTCTATTATTGTTTTAAGACCTCAGTAACCAT  2864

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GAAGATCACACATTCAGACTGTTGTGTCTGTGGAGTTTTAGGAGTGGGGGGTGACCTTTCTGGTCTTTGCACTTCCATC  3101

CTCTCCCACTTCCATCTGGCATCCCACCGGTTGTCCCCTGCACTTCTGGAAGGCACAGGGTGCTGCTGCCTCCTGGTCT  3180

TTGCCTTTGCTGGGCCTTCTGTGCAGGACGCTCAGCCTCAGGGCTCAGAAGGTGCCAGTCCGGTCCCAGGTCCCTTGTC  3259
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FIG. 2 (sheet 2 of 3)

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CCTTATATAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGGGSGGGGCCGC 3964

FIG. 2 (sheet 3 of 3)

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A Novel Domain within the 55 kd TNF Receptor Signals Cell Death

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Summary

Deletion mutagenesis of the intracellular region of the 55 kd TNF receptor (TNF-R1) identified an ~80 amino acid domain near the C-terminus responsible for signaling cytotoxicity. This domain shows weak homology with the intracellular domain of Fas antigen, a transmembrane polypeptide that can also initiate a signal for cytotoxicity. Alanine-scanning mutagenesis of TNF-R1 confirmed that many of the amino acids conserved with Fas antigen are critical for the cytotoxic signal. This region of TNF-R1-Fas homology is therefore likely to define a novel domain (death domain) that signals programed cell death. Mutations within the death domain of TNF-R1 also disrupted its ability to signal anti-viral activity and nitric oxide (NO) synthase induction. In addition, large deletions in the membrane-proximal half of the intracellular domain did not block signaling of cytotoxicity or anti-viral activity but did block induction of NO synthase.

Introduction

Tumor necrosis factor (TNF) is a cytokine produced mainly by activated macrophages. Although originally identified for its anti-tumor activity, TNF is now known to be one of the most pleiotropic cytokines, signaling a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of many genes (Goeddel et al., 1986; Beutler and Cerami, 1988; Old, 1988; Fiers, 1991). The first step in the induction of the various cellular responses mediated by TNF is its binding to specific cell surface receptors. Two distinct TNF receptors of ~55 kd (TNF-R1) and 75 kd (TNF-R2) have been identified (Hohmann et al., 1990; Brockhaus et al., 1990), and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990; Lewis et al., 1991; Goodwin et al., 1991).

The extracellular domains (ECDs) of human TNF-R1 and TNF-R2 share 28% sequence identity, approximately the same level of similarity they share with the ECDs of a number of diverse cell surface proteins, including the low affinity nerve growth factor (NGF) receptor, Fas antigen, CD40, OX40, and CD27 (Itoh et al., 1991; Camerini et al., 1991). Much of this sequence identity is a result of the extremely well conserved positions of cysteine residues that define this expanding receptor family. There is a complete absence of homology between the intracellular domains of the two TNF receptors, suggesting that they utilize distinct signaling pathways (Lewis et al., 1991). Also, with an exception noted below, the intracellular domains of the two TNF receptors do not show homology to other known proteins (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990; Lewis et al., 1991).

Numerous studies with anti-TNF receptor antibodies have demonstrated that TNF-R1 is the receptor that signals the large majority of the pleiotropic activities of TNF, including cytotoxicity, fibroblast proliferation, resistance to chlamidiae, synthesis of prostaglandin E_2, anti-viral activity, and manganese superoxide dismutase induction (Engelmann et al., 1990; Espevik et al., 1990; Tartaglia et al., 1991; Wong et al., 1992). Transfection-based assays for TNF-R1 have recently been developed by several groups (Tartaglia and Goeddel, 1992b; Brakebusch et al., 1992; Wiegmann et al., 1992), confirming the role of TNF-R1 in signaling cytotoxicity, anti-viral activity, and the stimulation of several second messenger pathways. Mutant TNF-R1s in which the majority of the intracellular domain has been removed are defective in initiating cytotoxicity, demonstrating the importance of this domain in mediating TNF signals (Tartaglia and Goeddel, 1992b; Brakebusch et al., 1992).

It has recently been shown that the Fas antigen, a member of the TNF/NGF receptor superfamily, can signal a programed cell death very similar to that mediated by TNF (Itoh et al., 1991). Fas antigen is involved in the negative selection of autoreactive T cells, and mice carrying a mutation in the intracellular domain of Fas antigen suffer from a dramatic autoimmune disorder (Watanabe-Fukunaga et al., 1992). In addition, a region of weak homology has been noted between the intracellular domains of TNF-R1 and Fas antigen (Itoh et al., 1991).

While the mechanism of programed cell death is not well understood, the importance of this process in biology is becoming increasingly apparent. The TNF-R1 transfection assay provides an ideal system for defining a signaling domain that can initiate programed cell death. Here we have defined an ~80 amino acid domain within TNF-R1 that can transmit a cytotoxic signal and that is also important in the signaling of other TNF activities. Furthermore, the spacing of this "death domain" relative to the ECD of TNF-R1 can be altered without loss of function.

Results

Delineating the TNF-R1 Death Domain

We showed previously that murine L929 cells expressing a transfected human TNF-R1 could be stimulated by agonist anti-human TNF-R1 antibodies (anti-hR1) to initiate a signal for cytotoxicity (Tartaglia and Goeddel, 1992b). Furthermore, a mutant TNF-R1 lacking the majority of its intracellular domain was defective in signal generation (Tartaglia and Goeddel, 1992b). To define better the se-

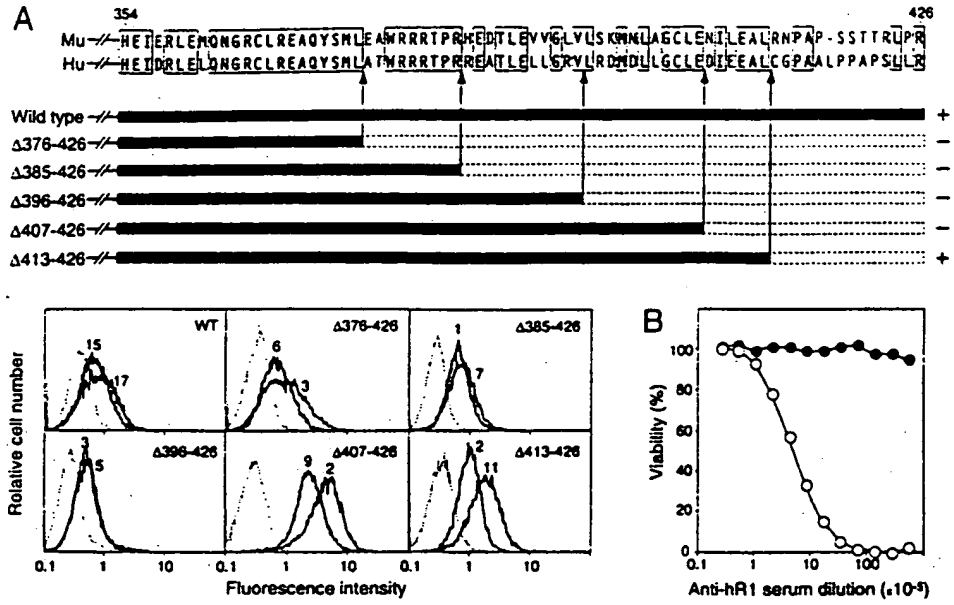

Figure 1. C-Terminal Truncations of TNF-R1

(A) Amino acid sequences of the C-terminal region of murine and human TNF-R1 (Lewis et al., 1991). Stippling indicates residues that are identical in the two species. Horizontal bars represent the C-terminus of mutant receptors, shaded regions indicate intact sequence, and dotted regions indicate deleted sequences. Expression of the mutant receptors in individual L929 clones was assessed by flow cytometric analysis. Shown are the expression profiles and clone numbers of the two individual L929 clones assayed in Table 1 in comparison with a L929-neo control clone (dotted curve). Cells were stained with anti-hTNF-R1 MAb 984 and phosphatidylethanolamine-conjugated goat anti-mouse immunoglobulins. Ten thousand cells were analyzed per sample. Histograms show relative cell number (y axis) versus log phosphatidylethanolamine fluorescence (x axis).

(B) Killing of L929 clones expressing TNF-R1s truncated for 14 and 20 C-terminal residues. Clones Δ413-426.11 (open circles) and Δ407-426.2 (closed circles) were treated with the indicated concentrations of anti-hR1 and 10 μg/ml cycloheximide for 24 hr. Cell viability was determined as described previously (Tartaglia and Goeddel, 1992b).

quences important for signaling cytotoxicity, we constructed expression vectors for a series of mutant receptors containing various C-terminal truncations (Figure 1A). The mutant receptor constructs were transfected into murine L929 cells, and several independent L929 clones expressing each of the mutant receptors were identified by flow cytometry (Figure 1A). At least two L929 clones expressing each of the mutant receptors were then assayed for a cytotoxic reaction in response to anti-hR1.

Mutant receptors in which 20 or more amino acids had been removed from the C-terminal end were found to be defective in signaling cytotoxicity, while a receptor truncated for the C-terminal 14 amino acids was still functional (Figure 1A and Table 1). The absolute importance of information contained between positions −14 and −20 is further illustrated in Figure 1B, in which the anti-hR1 sensitivities of L929 clones expressing the corresponding receptor mutants are compared in a dose response assay. These data indicate that the C-terminal extension of the cytotoxicity-signaling domain lies between positions −20 and −14.

To define the N-terminal extension of the cytotoxicity signaling domain, a series of TNF-R1 internal deletions were made and expressed in L929 cells. Several of these deletions removed large amounts of sequence in the membrane-proximal half of the intracellular domain without destroying the ability of TNF-R1 to signal cytotoxicity (Figure 2 and Table 1). The largest deletion that did not eliminate the cytotoxic signal extended from amino acid 212 (very close to the transmembrane region) to 326. In contrast, a deletion from amino acid 212 to 340 completely destroyed the ability of TNF-R1 to signal cytotoxicity. These results argue that the N-terminal extension of the cytotoxicity-signaling domain lies between positions 326 and 340 and that the C-terminal 100 amino acids of the intracellular domain contain sufficient information for independent folding and cytotoxicity signaling.

Homology to Fas Antigen

It has been noted previously that the intracellular domain of TNF-R1 shares a weak homology (29% identity over 45 amino acids) with the intracellular domain of Fas antigen (Itoh et al., 1991). Upon further inspection of these sequences, we noted that introduction of a 1 amino acid gap in the Fas sequence extended the region of homology an additional 20 amino acids (Figure 3). Although still not extensive, this putative homology was intriguing, because TNF-R1 and Fas antigen mediate a very similar cytotoxic reaction (Itoh et al., 1991). In addition, this region of homology falls within the boundaries of the TNF-R1 cytotoxicity-signaling domain defined by our deletion analysis, with the C-terminal limit of this homology falling exactly within the 6 amino acids that define the C-terminal extension of the cytotoxicity-signaling domain (Figure 3).

To further validate the relevance of the TNF-R1–Fas homology and also identify individual amino acids within TNF-R1 that are important in the cytotoxic signal, we initi-

Table 1. Signaling of Cytotoxicity by L929 Clones Expressing Human TNF-R1 Deletion Mutants

L929 Clone	Viability (%)
neo.3	98 ± 1
hR1.17	4 ± 1
hR1.15	3 ± 1
Δ376-426.3	100 ± 5
Δ376-426.6	102 ± 1
Δ385-426.1	97 ± 2
Δ385-426.7	96 ± 3
Δ396-426.3	101 ± 2
Δ396-426.5	90 ± 2
Δ407-426.9	94 ± 6
Δ407-426.20	101 ± 4
Δ413-426.11	2 ± 1
Δ413-426.2	10 ± 2
Δ258-308.16	17 ± 2
Δ258-308.19	12 ± 2
Δ212-308.2	7 ± 2
Δ212-308.29	4 ± 3
Δ258-326.9	15 ± 8
Δ258-326.24	45 ± 8
Δ212-326.2	55 ± 7
Δ212-326.5	43 ± 8
Δ212-340.20	98 ± 8
Δ212-340.21	101 ± 4

Two independent L929 clones were examined for each TNF-R1 deletion mutant. Cells were treated for 24 hr with a 1:400 dilution of agonist anti-human TNF-R1 antibody (anti-hR1) in the presence of 10 μg/ml cycloheximide. Values are expressed as percentage viability (± SD, n = 3) compared with the same cells treated with cycloheximide alone. L929 clone neo.3 is a G418-resistant control. hR1.17 and hR1.15 express the wild-type human TNF-R1 and have been referred to previously as L929.hR1.17 and L929.hR1.15 (Tartaglia and Goeddel, 1992b).

ated an alanine scanning mutagenesis of the TNF-R1 intracellular domain. Alanine was chosen as the replacement residue because it eliminates the side chain beyond the β carbon, yet does not alter the main chain conformation nor impose extreme electrostatic or steric effects (Cunningham and Wells, 1989). Many of the alanine substitutions were introduced at charged or aromatic residues that are conserved with the Fas antigen, although other amino acids were also mutagenized. In the first series of mutations, amino acids were altered in groups of 2 or 3. The information from these experiments provided the rationale for a mutagenesis of individual amino acids. All mutant receptors were stably expressed in L929 cells, and several independently isolated clones of each mutant were identified and examined in cytotoxicity assays. Table 2 summarizes the results of the entire substitution mutagenesis and shows cytotoxicity data for two representative clones expressing each receptor mutation.

Several outcomes of the TNF-R1 substitution mutagenesis are particularly noteworthy. First, many amino acids conserved between Fas and TNF-R1 were found to be essential for signaling cytotoxicity (Figure 3 and Table 2), validating the biological relevance of these similarities; in addition, changing Leu-351 to either Asn or Ala inhibited the cytotoxic signal of TNF-R1. Leu-351 in TNF-R1 corresponds to Ile-225 in murine Fas antigen, the position that when mutated to Asn is responsible for the lymphoproliferation (*lpr*) mutation in mice (Watanabe-Fukunaga et al., 1992). This provides additional evidence for a similar structure mediating cytotoxicity in both of these receptors. Second, critical residues were found scattered throughout the

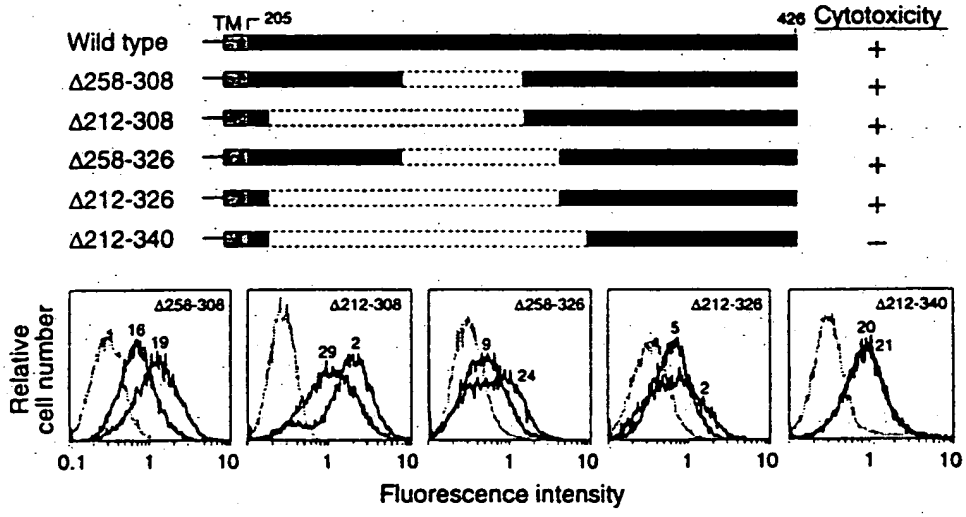

Figure 2. Internal Deletions within the TNF-R1 Intracellular Domain

The locations of deleted intracellular domain sequence in four mutant receptors are shown approximately to scale. Shaded regions indicate intact sequence; dotted regions indicate deleted sequences. Expression of the mutant receptors in individual L929 clones was assessed by flow cytometric analysis as in Figure 1. Shown are the expression profiles and clone numbers of the individual L929 clones assayed in Table 1 in comparison with a L929-neo control clone (dotted curve).

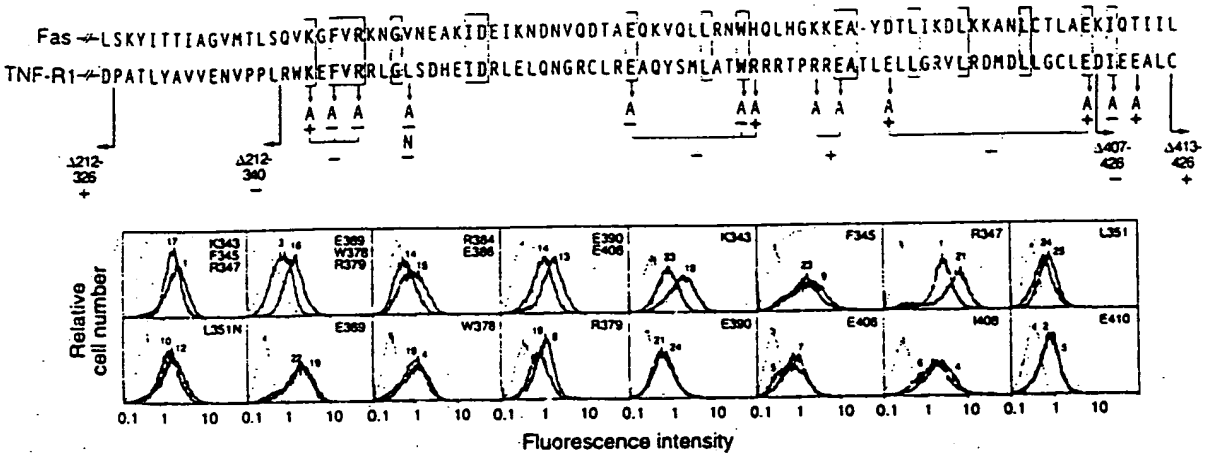

Figure 3. Substitution Mutagenesis of the Death Domain

The amino acid sequence of the TNF-R1 death domain (amino acids 326–413) is compared with the homologous region in the Fas antigen intracellular domain. Amino acids identical in the two sequences are stippled. The N-terminal and C-terminal deletions defining the death domain are indicated. Amino acids converted to alanine (A) by site-directed mutagenesis are indicated by an arrow pointing to the letter A. Leu-351 was also changed to Asparagine (N). A minus sign immediately below the letter A or N indicates that TNF-R1 could not signal cytotoxicity when this single amino acid replacement was made. A minus sign immediately below a bracket indicates that TNF-R1 could not signal cytotoxicity when the corresponding group of 2 or 3 amino acids was mutagenized. Expression of the mutant receptors in individual L929 clones was assessed by flow cytometric analysis as in Figure 1. Shown are the expression profiles of the individual L929 clones assayed in Table 2 in comparison with a L929-neo control clone (dotted curve).

65 amino acid homology region (from Phe-345 to Ile-408). This may suggest that a folded protein domain within TNF-R1 makes multiple noncontiguous contacts with other proteins involved in signal transduction. Third, the critical information between positions −14 and −20 (identified in the deletion analysis) may be the Ile that is conserved between Fas and TNF-R1, since substitution of only this conserved Ile results in a defective cytotoxic signal.

To determine whether the region within TNF-R1 that is responsible for signaling cytotoxicity is interchangeable with the corresponding region in the Fas antigen, a TNF-R1–Fas antigen fusion was generated and expressed in L929 cells. The fusion protein contains TNF-R1 sequences from position 1 to 323 fused to Fas sequences from position 210 to 319. This fusion therefore replaces TNF-R1 sequences beginning 3 amino acids before the N-terminal extension of the death domain with the corresponding Fas antigen sequences. The position of this fusion junction also results in a precise swap of amino acids encoded on the final exons of the TNF-R1 and Fas antigen genes (Fuchs et al., 1992; S. Nagata, personal communication). Three independently isolated L929 clones expressing this fusion protein showed modest cytotoxicity in response to anti-hR1 (Table 2), further demonstrating the relevance of the homology between these two proteins.

Positional Flexibility of Death Domain Relative to ECD

Considerable evidence has accumulated that the mechanism of TNF-R1 triggering involves the cross-linking of receptors by the TNF ligand (reviewed by Tartaglia and Goeddel, 1992a). It has also been demonstrated that the association of intracellular domains is critical in signal generation (Tartaglia and Goeddel, 1992b). It therefore surprised us when large internal deletions between the transmembrane region and the death domain did not interfere with signaling. These internal deletions might be expected to destroy the register between the ligand cross-linked ECDs and the associating intracellular domain sequences. Therefore, information contained in the orientation of TNF-R1 cross-linking would be lost upon removal of internal sequences and could potentially interfere with proper association of the intracellular domains. However, a possible explanation for the positional flexibility of the death domain relative to the ECD in our experiments is that polyclonal antibodies were used as ligand. These antibodies may cross-link the TNF-R1 molecules in a variety of orientations. We tested whether TNF itself could trigger cytotoxicity through mutant receptors with altered spacing between the ECDs and the death domain. This required blocking of the endogenous murine TNF-R1 on the L929 clones by pretreatment with an antagonist monoclonal antibody (MAb) against murine TNF-R1. These cells were then treated with TNF (which now has access only to the transfected human TNF-R1) and assayed for cytotoxicity. L929 cells expressing either the wild-type human TNF-R1 or mutant TNF-R1s with large deletions between the ECD and the death domain were all sensitive to TNF, even after access to the endogenous murine TNF-R1 was blocked (Figure 4). This indicates that the death domain has positional flexibility relative to the TNF cross-linked ECDs and suggests that information contained in the orientation of receptor aggregation may not be critical in signal generation.

Effect of TNF-R1 Mutations on the Signaling of Antiviral Activity and the Induction of Nitric Oxide Synthase

TNF-R1 is known to signal a large number of diverse biological activities in addition to cytotoxicity. We thus were

Table 2. Signaling of Cytotoxicity by Human TNF-R1 Mutants

L929 Clone	Viability (%)
neo.3	95 ± 2
hR1.17	4 ± 1
hR1.15	3 ± 1
K343, F345, R347.1	99 ± 5
K343, F345, R347.17	102 ± 3
E369, W378, R379.3	92 ± 4
E369, W378, R379.16	96 ± 5
R384, E386.14	3 ± 1
R384, E386.15	30 ± 5
E390, E406.13	97 ± 6
E390, E406.14	96 ± 3
K343.18	22 ± 5
K343.23	43 ± 2
F345.9	90 ± 2
F345.23	87 ± 16
R347.1	88 ± 4
R347.21	94 ± 7
L351.24	109 ± 5
L351.25	97 ± 2
L351.10→N	101 ± 8
L351.12→N	97 ± 4
E369.19	80 ± 7
E369.22	93 ± 2
W378.19	92 ± 8
W378.4	88 ± 6
R379.8	16 ± 3
R379.19	2 ± 2
E390.21	75 ± 4
E390.24	47 ± 5
E406.5	30 ± 1
E406.7	50 ± 10
I408.4	86 ± 3
I408.6	87 ± 9
E410.2	21 ± 2
E410.5	2 ± 1
R1–Fas.3	59 ± 9
R1–Fas.9	68 ± 7

Two independent L929 clones were examined for each TNF-R1 mutant. Cells were treated for 24 hr with a 1:400 dilution of anti-hR1 polyclonal antibody in the presence of 10 μg/ml cycloheximide. Values are expressed as percentage viability (± SD, n = 3) compared with the same cells treated with cycloheximide alone. All listed mutations were alanine substitutions unless otherwise indicated.

interested to learn whether mutations that affected the signaling of cytotoxicity altered the signaling of other TNF activities. We have recently shown that the known anti-viral activities of TNF are signaled by TNF-R1, and that human TNF-R1 (together with interferon γ [IFN-γ]) can transmit an anti-viral signal when expressed in murine L929 cells (Wong et al., 1992). We therefore assayed mutant human TNF-R1s expressed in L929 cells for their ability to transmit a signal that results in protection from subsequent infection by vesicular stomatitis virus (Table 3). All mutations tested within the death domain that blocked the signaling of cytotoxicity also eliminated the signaling of anti-viral activity, while the mutations that did not eliminate the cytotoxic signal also did not interfere with the anti-viral signal. In addition, the large deletions between the ECD and death domain that did not block the signaling of cytotoxicity also did not block the signaling of anti-viral activity. This inability to separate the signaling of cytotoxicity and anti-viral activity by the many TNF-R1 mutations suggests

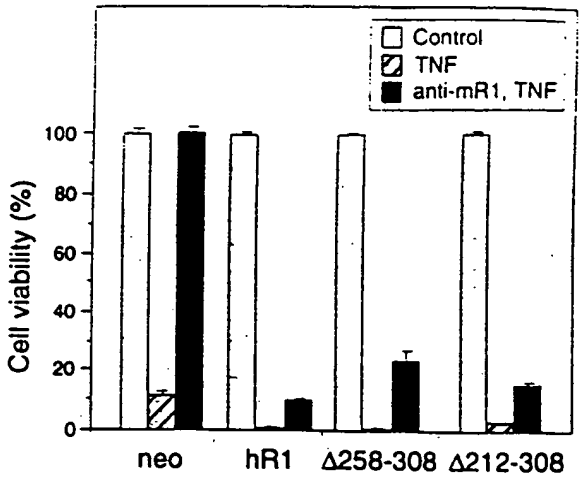

Figure 4. TNF Killing of L929 Cells Expressing TNF-R1s with Altered Spacing between the ECD and Death Domain

A control L929 clone (neo.5) and L929 clones expressing wild-type (hR1.17) or mutant TNF-R1s (Δ258-308.16 and Δ212-308.2) were pretreated for 1 hr with a 1:10 dilution (hybridoma supernatant) of anti-murine TNF-R1 MAb 176. Cells were then further treated with 100 ng/ml TNF for 24 hr in the absence of cycloheximide. Cell viability was determined as described previously (Tartaglia and Goeddel, 1992b).

that a common signal from TNF-R1 initiates both the cytotoxic and anti-viral programs.

Another important biological activity mediated by TNF in combination with IFN-γ is the induction of a nitric oxide (NO) synthase activity (Farrar et al., 1992). Preliminary experiments with agonist antibodies to murine TNF-R1 and TNF-R2 showed that in L929 cells this TNF activity was mediated by murine TNF-R1 (data not shown). In addition, human TNF-R1 could initiate NO induction in L929 cells that were transfected with wild-type human TNF-R1 (see below). To determine whether the same mutations that interfere with the signaling of cytotoxicity and anti-viral activity also interfere with the induction of NO synthase, we assayed L929 clones expressing the mutant human TNF-R1s for NO synthase induction in response to anti-hR1 and murine IFN-γ (mIFN-γ). As shown in Table 3, mutations within the death domain of TNF-R1 that are negative for cytotoxicity are also negative for NO synthase induction. The negative data obtained for these mutant receptors are not due to the inability of the host L929 clones to induce NO synthase, because these clones could still induce NO through the endogenous murine TNF-R1 in response to either anti-murine TNF-R1 antibodies or TNF itself (data not shown). Mutations within the death domain that did not block the signaling of cytotoxicity also did not block the signaling of NO synthase induction. Therefore, the signal initiated from the death domain that is responsible for cytotoxicity may also be required for NO synthase induction. Interestingly, all of the large deletions between the transmembrane domain and the death domain eliminated the signaling of NO synthase induction (Table 3). The signaling of NO synthase induction thus appears to require both an intact death domain and additional sequences in the N-terminal half of the intracellular domain.

Table 3. Summary of TNF-R1 Mutational Analysis

Receptor Mutation	Signaling		
	Cytotoxicity	Antiviral	NO Synthase Induction
Wild type	+	+	+
Δ245-426	–	–	–
Δ376-426	–	ND	ND
Δ385-426	–	ND	ND
Δ396-426	–	ND	ND
Δ407-426	–	–	–
Δ413-426	+	+	+
K343, F345, R347→A	–	–	–
K343→A	+	+	+
F345→A	–	–	–
R347→A	–	–	–
L351→A	–	–	–
L351→N (lpr)	–	–	–
E349, W378, R379→A	–	ND	–
E369→A	–	–	–
W378→A	–	–	–
R379→A	+	+	+
R384, E386→A	+	+	+
E390, E406→A	–	–	–
E390→A	+/–	+	+
E406→A	+	+	+
I408→A	–	–	–
E410→A	+	+	+
Δ258-308	+	+	–
Δ258-326	+	+	–
Δ212-308	+	+	–
Δ212-326	+	+	–
Δ212-340	–	–	ND

For each of the indicated mutations, at least two independently isolated L929 clones expressing the corresponding mutation were analyzed. Triplicate determinations were made on each L929 clone for the cytotoxicity (see Tables 1 and 2) and NO assays, and sextuplicate determinations were made for the antiviral assay. A plus sign in the cytotoxicity assay indicates greater than 50% cytotoxicity under the conditions described in Tables 1 and 2, while a minus sign indicates less than 20% cytotoxicity. A plus sign in the antiviral assay indicates greater than 75% survival under the conditions described in Experimental Procedures, while a minus sign indicates less than 25% survival. In the antiviral assay, agonist antibody activation of the wild-type receptor in hR1.17 cells resulted in 80% ± 2% survival. Survival in untreated cells was typically between 15% and 20%. The reliable detection limit of the NO synthase assay was 0.3 nmol of nitrite per 10^5 cells under the conditions described in Experimental Procedures. A minus sign indicates that nitrite levels measured after induction were below the reliable detection limit. A plus sign indicates that nitrite levels were induced to greater than 0.8 nmol per 10^5 cells. In the NO assay, agonist antibody activation of the wild-type receptor in hR1.17 cells resulted in a measured nitrite level of 2.6 ± 0.2 nmol per 10^5 cells. ND, not determined.

A Subset of TNF-R1 Negative Mutations Have Dominant Negative Character

In a previous report, we demonstrated that expression in L929 cells of a truncated human TNF-R1 (missing the majority of its intracellular domain) resulted in suppressed signaling by the endogenous murine TNF-R1 (Tartaglia and Goeddel, 1992b). This dominant negative effect was due to TNF cross-linking the functional endogenous receptors to the nonfunctional truncated receptors. The resulting receptor complexes lacked interacting intracellular domains and were therefore defective in signal generation (Tartaglia and Goeddel, 1992b; Brakebusch et al., 1992). As described above, we have now identified a number of negative mutations within human TNF-R1 that result from only minor changes in intracellular domain sequence. We were now interested whether these less extensive mutations would also act as dominant negative mutations.

As revealed by previous work (Tartaglia and Goeddel, 1992b) and our preliminary experiments, an assessment of the dominant negative character of a mutant transmembrane receptor is complicated by two factors: first, the extent of the dominant negative effect is highly dependent on the expression level of the mutant receptor; and second, even mutant receptors that do not have true dominant negative character can appear to reduce the sensitivity of a cell to TNF in a dose response assay if the ligand is titrated from the assay media. To circumvent the first problem, we restricted our analysis to only those few cell lines in which the expression level of human TNF-R1 was equal to or greater than that in a well-characterized control cell line (L929.hR1Δ.4; Tartaglia and Goeddel, 1992b). This control line expresses a truncated TNF-R1 at a level that significantly suppresses signaling by the endogenous murine TNF-R1. To circumvent the problem of ligand titration from the assay media, we examined the TNF sensitivity of L929 clones under conditions of extreme ligand excess (50 nM) and in the absence of cycloheximide. Several of the TNF-R1 mutations clearly acted as dominant negative mutations, as evidenced by the decreased TNF sensitivity of the corresponding L929 clones (Table 4). As shown previously for the L929.hR1Δ.16 clone (Tartaglia and Goeddel, 1992b), the decreased sensitivity of these clones was not due to differences in murine TNF-R1 levels or the signal transduction apparatus, because pretreatment with a human TNF-R1 antagonist antibody restored normal sensitivity (data not shown). Interestingly, several of the cytotoxicity-signaling negative mutations did not act as dominant negative mutations, despite high level receptor expression. There was a clear pattern in the intracellular domain location of these mutations. Mutations that disrupted the N-terminal half of the death domain had dominant negative character, while those that disrupted information in the C-terminal half of the death domain did not.

Discussion

Despite the absence of recognizable signaling motifs or kinase homologies, the 55 kd TNF receptor (TNF-R1) signals a large number of diverse biological activities. Of particular interest is its ability to initiate a rapid cytotoxic program, since the signaling mechanisms of programed cell death are poorly understood. To understand better the mechanisms by which TNF-R1 signals cell death and other important biological responses, we have begun to define sequences within its intracellular domain that are required for function.

Through a series of both C-terminal truncations and internal deletions, we have identified an ~80 amino acid domain within the 221 amino acid intracellular region of TNF-R1 that is required and sufficient for initiating the signal for cytotoxicity. The C-terminal extension of this domain is close to the C-terminus of the receptor (removal

Table 4. Dominant Negative Character of Human TNF-R1 Mutations

L929 Clone	Viability (%)
neo.3	1.7 ± 0.5
Δ245-426.4	55.7 ± 2.3
Δ212-340.8	78.9 ± 4.7
K343, F345, R347.1	21.2 ± 1.0
F345.9	33.4 ± 1.0
R347.1	35.4 ± 0.5
R347.21	50.3 ± 2.2
E390, E406.13	5.7 ± 2.1
I408.4	6.4 ± 0.5
Δ407-426.2	1.8 ± 0.6
Δ407-426.9	2.0 ± 0.4

Cells were treated for 18 hr with 50 nM TNF. Values are expressed as percentage viability (± SD, n = 6) compared with controls. Clone Δ245-426.4 was referred to previously as L929.hR1Δ.4 (Tartaglia and Goeddel, 1992b).

of the terminal 20 but not the terminal 14 amino acids eliminates signaling), and the N-terminal extension is near the center of the intracellular domain primary sequence. Sequence information within the N-terminal half of the intracellular domain does not appear to be required for the cytotoxic signal. The results of both the C- and N-terminal deletion analysis are very consistent with the homology between the mouse and human TNF-R1s (Lewis et al., 1991). Strong conservation between the murine and human TNF-R1s begins very close to the N-terminal extension of the death domain. In addition, this homology drops off abruptly in the C-terminal 14 amino acids.

The 80 amino acid cytotoxicity-signaling domain (death domain) within TNF-R1 contains a region of 65 amino acids that shows 28% identity to a region within the intracellular domain of Fas antigen. Although this homology is not extensive, the Fas antigen can signal a programed cell death very similar to that signaled by TNF-R1 (Itoh et al., 1991), thus establishing a functional conservation between the two receptors. To test the relevance of the amino acid sequence homology, we performed alanine scanning mutagenesis on amino acids within the intracellular domain of TNF-R1 that are conserved with the Fas antigen. This revealed a number of residues within TNF-R1 that are critical for TNF-R1 function, further validating the significance of the homology between the intracellular domains of these two receptors. A number of these essential residues are charged and so are likely to be exposed on the surface of the death domain. These residues may thus represent positions at which the TNF-R1 intracellular domain interacts with cytoplasmic proteins or possibly other TNF-R1 intracellular domains. The large number of such essential residues scattered throughout a region of ~65 amino acids suggests that TNF-R1 does not display a short peptide that binds and stimulates an intracellular signaling component, but rather makes multiple noncontiguous contacts with a folded structural domain. However, our experiments do not rule out the possibility that some of the amino acid replacements may disrupt proper folding of the intracellular domain. Whether Fas and TNF-R1 interact with common or different intracellular signaling proteins remains to be determined. It is possible that the relatively weak homology (28% identity) reflects only a conserved structural framework between these two proteins and that nonidentical residues provide the specificity for interaction with distinct factors.

Mutant TNF-R1s containing large internal deletions were still able to generate a cytotoxic signal, demonstrating that the spacing between the death domain and the ECD can be altered without loss of signaling. This is surprising, as TNF-R1 triggering requires the association of multiple intracellular domains upon ligand- or antibody-induced aggregation of TNF-R1 molecules (Tartaglia and Goeddel, 1992b). The insensitivity to altered spacing implies that the register between the ECD and the death domain is flexible enough to accommodate repositioning to allow proper intermolecular association of intracellular domains within a ligand cross-linked complex. This positional flexibility indicates that the death domain is both an independent functional and a structural domain. Interestingly, the largest deletion that did not completely block cytotoxicity (Δ212-326) extends only 3 amino acids into the sequence (amino acids 324–426) encoded by the final exon of the TNF-R1 gene. When amino acids 324–426 of TNF-R1 (which contain necessary and sufficient information for the cytotoxic signal) were replaced by the corresponding Fas antigen sequences, the resulting receptor chimera was able to generate a modest cytotoxic signal. These results further support a modular structure of the death domain and a relationship with the corresponding region in the Fas antigen.

TNF-R1 initiates signals for a large number of biological activities in addition to cytotoxicity. To examine how mutations within the death domain affected the signaling of other TNF activities, we assayed the ability of mutant TNF receptors to initiate signals for anti-viral activity and the induction of NO synthase. The signaling of cytotoxicity and anti-viral activity proved to be mutationally inseparable within the group of mutations analyzed in our study. Given the large number of mutations analyzed, these data suggest that a common signal is required to initiate both the anti-viral and cytotoxicity programs. Mutations within the death domain that disrupted the ability of TNF-R1 to signal for the induction of NO synthase were identical to those that disrupted the signaling of anti-viral activity and cytotoxicity. Interestingly, deletion mutations in the membrane-proximal half of the intracellular domain also disrupted the ability of TNF-R1 to signal for NO induction, even though the loss of this information did not eliminate the signaling of anti-viral activity or cytotoxicity. This implies that the induction of NO synthase requires the same signaling information required for cytotoxicity and anti-viral activity as well as additional sequence or spacing information in the membrane-proximal half of the intracellular domain. Thus, the signaling of a subset of TNF activities may require more signaling information than that initiated by the death domain, demonstrating that it is possible mutationally to separate distinct TNF activities at the level of signal initiation from the intracellular domain. The requirement of only the death domain for some activities and additional information for other activities is roughly paralleled in the interleukin 2 receptor system (Hatake-

yama et al., 1992). The interleukin 2 β chain requires only its serine-rich domain to signal for proliferation in BAF3 cells, yet c-*fos* induction requires both the serine-rich domain and an additional region.

The triggering of TNF-R1 is a consequence of the cross-linking of TNF-R1 monomers by either anti-receptor antibodies or the TNF trimer (reviewed by Tartaglia and Goeddel, 1992a). The analysis discussed above has defined amino acids that, when mutated in all chains within a homoreceptor complex, destroy the ability to function. However, we were also interested in distinguishing between intracellular domain information that is required in all TNF-R1 molecules and information that is more redundant in the receptor complex. An analysis of the dominant negative character of a mutant transmembrane receptor should provide insight into this. Those receptors mutated for intracellular domain information that is required in all receptor chains within a complex should suppress signaling when cross-linked to functional receptors. Alternatively, mutations in information not required in all receptor chains would not be expected to have strong dominant negative character upon their cross-linking to intact receptors.

Our analysis of several inactive TNF-R1s identified both classes of mutations: those that had dominant negative character and those that did not. The differences observed between these two classes were not a consequence of expression level, but rather were a function of the location at which the mutation occurred in the intracellular domain. Those mutations that disrupted information in the N-terminal half of the death domain had dominant negative character, while those that disrupted information in the C-terminal half did not. One possible explanation for these two phenotypes is that sequences in the N-terminal half of the death domain are involved in intracellular domain association. Therefore, when the TNF ligand cross-links such mutant receptors to wild-type receptors, proper intracellular domain association does not occur, and the receptor complex cannot be activated for signaling. In contrast, sequences in the C-terminal half may not be critical for intracellular domain association and the subsequent conversion to an active complex; rather, they may associate with intracellular signaling molecules, and the association of every receptor chain with a signaling molecule may not be essential for the generation of a signal.

A more complete understanding of the signaling of programed cell death will require both structural studies on the death domains of TNF-R1, Fas antigen, and other similar molecules and the identification of intracellular molecules that interact with them. It will also be of interest to learn whether there exists a family of intracellular killing proteins that provides the specificity for programed cell death in different tissues. This information might provide the tools to manipulate cell death both positively and negatively for the treatment of many disease states.

Experimental Procedures

Reagents

Recombinant human TNF (of specific activity over 10^7 U/mg) and mIFN-γ were provided by the Genentech manufacturing group. The rabbit anti-murine TNF-R1 and rabbit anti-hR1 polyclonal agonist antibodies have been described previously (Tartaglia and Goeddel, 1991; Tartaglia and Goeddel, 1992b). The titer of the anti-hR1 was 1:150,000, as quantitated by a direct antigen-coated enzyme-linked immunosorbent assay. MAb 984 against human TNF-R1 inhibits the binding of TNF to human TNF-R1 and has also been described previously (Tartaglia and Goeddel, 1992b). MAb 176 against murine TNF-R1 inhibits the binding of TNF to murine TNF-R1 (Tartaglia et al., 1993).

TNF-R1 Mutagenesis

The starting plasmid for the TNF-R1 mutagenesis contained the cDNA encoding wild-type TNF-R1 cloned into the Rous sarcoma virus long terminal repeat expression vector pRIS and has been described previously (Tartaglia and Goeddel, 1992b). Plasmids encoding human TNF-R1s with C-terminal truncations were generated by replacement of sequences between the HindIII restriction site and convenient restriction sites with synthetic DNA containing an in-frame stop codon. Verification of correctly modified cDNAs was determined by double-strand DNA sequencing. All internal deletions and substitution mutations were generated by site-directed mutagenesis as follows. Regions of the TNF-R1 to be mutated were subcloned into Bluescript SK(+) (Stratagene) and made single stranded in the *dut⁻ ung⁻* F' Escherichia coli CJ236 strain. Oligonucleotides that contained the desired mutation and were complementary to the single-stranded template were used for primer extension (Kunkel et al., 1987). The primer-extended product was transformed into DH5αF'. Following DNA sequencing to confirm the sequence of the mutated region, the TNF-R1 fragment was subcloned back into the pRIS expression vector.

Generation of Murine L929 Clones Expressing Human TNF-R1

The expression vectors encoding the mutant human TNF-R1s were introduced into mouse L929 cells by electroporation. Cells (5×10^6 in 1.0 ml) were cotransfected with 0.5 μg of ScaI-digested pRK.neo and 20 μg of ScaI-digested TNF-R1 expression vector. Cells were plated into 15 cm plates and, after 2 days, selected in medium containing 600 μg/ml G418. After 12 days, individual G418-resistant clones were picked and expanded. To examine the expression of human TNF-R1, cells were incubated on ice for 60 min with 100 μg/ml anti-hR1 MAb 984 in phosphate-buffered saline containing 2% fetal bovine serum. The cells were then washed and stained with phosphatidylethanolamine-conjugated goat anti-mouse immunoglobulins (Caltag Laboratories) and analyzed on an Epics Elite instrument (Coulter Electronics).

L929 Cytotoxicity Assay

L929 cells (2×10^4 per well) were seeded into 96-well microtiter plates in 100 μl of medium (low glucose Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal calf serum, 1% L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin; GIBCO) and incubated for 24 hr at 37°C in a 5% CO_2 atmosphere. The medium was then brought to 10 μg/ml cycloheximide, and the anti-hR1 or TNF was added to the wells and serially diluted. The plates were incubated for an additional 24 hr (or 18 hr for the dominant negative assay) and the viable cells stained with 20% methanol containing 0.5% crystal violet. The dye was eluted with 0.1 M sodium citrate/0.1 M citric acid and 50% ethanol, and absorbance was measured at 540 nm.

NO Assay

Cells were seeded at 1×10^5 cells/ml in Corning 24-well tissue culture plates, incubated at 37°C for 24 hr, and then treated with mIFN-γ (50 U/ml), alone or in combination with TNF (100 ng/ml), anti-hR1 (1:400 dilution), or anti-mR1 (1:1000 dilution). After 48 hr at 37°C, supernatants were assayed for nitrite by the Greiss reaction (Green et al., 1982).

Antiviral Assay

A suspension of cells (100 μl) at 2×10^5/ml in DMEM supplemented with a 5% fetal calf serum was added to each well of a 96-well plate for 24 hr before the assay. Anti-hR1 was then added to the attached cells at a 1:400 dilution in combination with 0.1 ng/ml mIFN-γ. After 24 hr, cells were challenged with vesicular stomatitus virus diluted in DMEM with 2% fetal calf serum at a multiplicity of infection of 0.1 and were further incubated at 37°C. After 24 hr, virus control wells were checked by microscopic examination to confirm >80% lysis. The fluid

from all wells was poured off, and the attached viable cells were stained with 0.5% crystal violet in 20% methanol for 15 min at ambient temperature. Cell viability was determined by eluting the dye from the stained cells with 0.1 M sodium citrate/0.1 M citric acid and 50% ethanol and measuring absorption at 540 nm. No anti-viral activity was mediated by mIFN-γ alone under the conditions of this assay.

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CAR1, a TNFR-Related Protein, Is a Cellular Receptor for Cytopathic Avian Leukosis–Sarcoma Viruses and Mediates Apoptosis

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Summary

Viral envelope (Env)–receptor interactions have been implicated in the cell death associated with infection by subgroups B and D avian leukosis–sarcoma viruses (ALVs). A chicken protein, CAR1, was identified that permitted infection of mammalian cells by these viral subgroups. CAR1 bound to a viral Env fusion protein, comprising an ALV-B surface Env protein and the Fc region of an immunoglobulin, indicating that it is a specific viral receptor. CAR1 contains two extracellular cysteine-rich domains characteristic of the TNFR family and a cytoplasmic region strikingly similar to the death domain of TNFR1 and Fas, implicating this receptor in cell killing. Chicken embryo fibroblasts susceptible to ALV-B infection and transfected quail QT6 cells expressing CAR1 underwent apoptosis in response to the Env-Ig fusion protein, demonstrating that this cytopathic ALV receptor can mediate cell death.

Introduction

Avian leukosis–sarcoma viruses (ALVs) are divided into cytopathic and noncytopathic subgroups. In contrast to infection by noncytopathic viral subgroups (A, C, and E), which does not lead to cell killing, infection by cytopathic viral subgroups (B, D, and F) can lead to the death of as much as 30%–40% of target cells during the acute phase of infection (Weller et al., 1980; Weller and Temin, 1981). Cells killed by cytopathic ALVs contain approximately 300–400 copies of unintegrated viral DNA per cell, whereas noncytopathic ALV infections do not lead to accumulation of unintegrated viral DNA (Weller et al., 1980; Weller and Temin, 1981). Also, addition of antisera to prevent viral reinfection abrogates the viral cytopathic effect (CPE; Weller et al., 1980). These observations have led to a proposal that the cell killing associated with cytopathic ALV infections might be due to massive viral superinfection (Weller et al., 1980; Weller and Temin, 1981). Indeed, infected cells that survive this transient period of cell killing are resistant to viral superinfection because the cognate receptors are functionally down-regulated (reviewed by Weiss, 1993). Multiple rounds of viral infection have also been implicated in the CPE associated with other retroviruses, including HIV-1 (Somasundaran and Robinson, 1987; Stevenson et al., 1988; Pauza et al., 1990; Robinson and Zinkus, 1990), spleen necrosis virus (Keshet and Temin, 1979), and the feline leukemia virus FeLV-FAIDS (Donahue et al., 1991). In the case of HIV-1, however, superinfection is not required for cell killing (Bergeron and Sodroski, 1992; Laurent-Crawford and Hovanessian, 1993).

CPE determinants have been mapped to the envelope (Env) glycoproteins of several retroviruses, including ALV-B (Domer and Coffin, 1986), HIV-1 (reviewed by Siliciano, 1996), avian hemangioma virus (Resnick-Roguel et al., 1989), Cas-Br-E–murine leukemia virus (Paquette et al., 1989), feline leukemia virus C (Riedel et al., 1988), and the feline leukemia virus FeLV-FAIDS (Donahue et al., 1991). The CPE determinants of the ALV-B Env subunit surface (SU) also specify usage of the subgroup B viral receptor on chicken cells (Domer and Coffin, 1986). This observation suggests that Env–receptor interactions might contribute directly to cell killing.

Cytopathic ALV-B and ALV-D and noncytopathic ALV-E are predicted to share a cellular receptor encoded by the chicken *tv-b* locus (reviewed by Weiss, 1993). A set of five different alleles of *tv-b* has been identified: *tv-b*^{1}, *tv-b*^{2a}, and *tv-b*^{2b} permit infection by all three viral subgroups; *tv-b*^{3} allows infection by ALV-B and ALV-D, but not ALV-E; and *tv-b*^{r} does not permit entry by any of these viruses (reviewed by Weiss, 1993).

To understand the mechanism of cell killing induced by cytopathic subgroups of ALV, we have cloned a chicken gene, presumably *tv-b*^{3}, which encodes a cellular receptor for the subgroup B and D viruses. This receptor is a member of the tumor necrosis factor receptor (TNFR) family, and interaction of this protein with a subgroup B SU–Ig fusion protein promotes the death of avian cells. This result indicates that cytopathic ALV Env–receptor interactions might contribute directly to virus-induced cell death.

Results

Isolation of Genomic and cDNA Clones That Permit Infection by Cytopathic Subgroups of ALV

A gene transfer approach was used in an attempt to isolate the chicken *tv-b*^{3} locus, predicted to encode cellular receptors for the cytopathic subgroups B and D ALV (Weiss, 1993). Mouse 3T3 cells were cotransfected with pMPHis plasmid DNA conferring histidinol resistance (Young et al., 1993) and with genomic DNA from chicken embryo fibroblasts (CEFs) homozygous for *tv-b*^{3}. To identify which of the approximately 3,300 histidinol-resistant colonies were susceptible to ALV-B infection, we challenged them with the subgroup B–specific virus RCASH-B encoding hygromycin B phosphotransferase (Young et al., 1993). The resultant 19 hygromycin B–resistant colonies were challenged with another subgroup B–specific virus, RCASB-Neo, conferring resistance to G418. A single primary transfectant, designated 11B, was infected by both subgroup B viruses. The presence of approximately six copies of RCASH-B proviral DNA in the population of cells derived from transfectant

11B confirmed the occurrence of multiple infection events, consistent with receptor-mediated viral entry (data not shown). Southern blot analysis (Southern, 1975) demonstrated the presence of chicken genomic DNA in this transfectant (data not shown), as it contained multiple copies of B5/B6 avian repeat DNA (Stumph et al., 1981).

To segregate the putative ALV-B susceptibility gene from other transfected DNA sequences, we performed a second round of transfection and selection. Genomic DNA from primary transfectant 11B was cotransfected with pPur plasmid DNA encoding puromycin N-acetyltransferase into mouse 3T3 cells. The resulting 20,000 colonies were challenged with RCASB-Neo and RCASH-B, leading to 14 colonies resistant to G418 and hygromycin B.

The primary transfectant 11B contained multiple copies of pMPHIS plasmid DNA (data not shown), raising the possibility that plasmid sequences were linked to the ALV-B susceptibility locus. Indeed, Southern blot analysis demonstrated that 7 of 12 secondary transfectants contained a shared locus with a unique copy of pMPHIS plasmid DNA (Figure 1A). Thus, this plasmid DNA appeared to be linked to the susceptibility locus and was used as a molecular tag for cloning.

To isolate the chicken DNA sequences linked to this plasmid, a genomic DNA library prepared from secondary transfectant C12 was screened by hybridization with a probe specific for the histidinol dehydrogenase gene. The BK-1c clone was isolated (Figure 1A), and derivative restriction fragments were used as hybridization probes to isolate the overlapping genomic DNA clone BK-9 (Figure 1A). Southern blot analysis using probes derived from both of these clones revealed a 7.5 kb stretch of chicken genomic DNA shared between several independent secondary transfectants (Figure 1A). Human 293 cells transfected with the BK-9 genomic DNA clone could be infected by RCASH-B, indicating that the region of the shared chicken locus contained within this clone encodes the ALV-B susceptibility factor (data not shown).

To identify RNA transcripts encoding this factor, Northern blot analysis was performed using three probes derived from the shared region. A single 2.3 kb RNA transcript hybridized with all three probes (Figure 1B). Therefore, a cDNA library constructed from CEFs was screened with these probes. One clone, designated 7.6-2, which hybridized with all three probes, was isolated and conferred susceptibility to ALV-B infection upon COS-7 cells (Figure 1C). Furthermore, this clone increased by approximately 10-fold the susceptibility of these cells to infection by subgroup D viruses (Figure 1C), which are moderately mammal-tropic (Bova et al., 1988). These results indicate that the cloned gene is probably *tv-b*^{s3}, predicted to encode a factor allowing infection specifically by ALV-B and ALV-D (Weiss, 1993). As expected, the transfected COS-7 cells were not efficiently infected by subgroup A, C, or E viruses (Figure 1C), which do not use receptors encoded by this allele of *tv-b* (Weiss, 1993).

Preliminary studies have demonstrated that the candidate *tv-b* gene may be expressed ubiquitously in chicken tissues. Northern blot analysis using the 7.6-2 cDNA clone as a probe revealed an approximately 2.3 kb RNA species in several different chicken tissues including bursa, gizzard, liver, heart, and lung (data not shown). However, these transcripts appear to be much less abundant than those observed in CEFs.

The Cloned Factor Binds Specifically to the ALV-B SU Protein

The 7.6-2 cDNA clone was sequenced leading to the identification of a single long open reading frame that appears to encode a type I membrane protein with an estimated molecular mass of 39 kDa. This protein is predicted to contain a signal peptide, an extracellular domain with two putative N-linked glycosylation sites, a single transmembrane region, and a long cytoplasmic tail (Figure 2).

To test whether the cloned factor bound specifically to ALV-B Env, an immunoadhesin (SUB–rIgG) comprising a subgroup B SU protein fused to the Fc region of a rabbit immunoglobulin was constructed. The SUB–rIgG protein migrated at approximately 200 kDa under nonreducing conditions on an SDS–polyacrylamide gel (Figure 3A) but at approximately 120 kDa when reduced (data not shown). This is consistent with the formation of disulfide-linked homodimers similar to those of other immunoadhesins (Capon et al., 1989; Haak-Frendscho et al., 1993; Pitti et al., 1994). The SUB–rIgG protein precipitated two predominant proteins (approximately 42 kDa and 45 kDa in size) from lysates of human 293 cells transfected with the 7.6-2 cDNA clone (Figure 3B, right panel), but not from cells expressing Tva, the ALV-A receptor (Figure 3B, left panel). These two proteins were not precipitated by a control immunoadhesin (SUA-rIgG) containing the SU portion of a subgroup A Env protein, which instead precipitated the heterogeneously modified Tva proteins (Bates et al., 1993; Figure 3B). Endoglycosidase H digestion of the 42 kDa and 45 kDa proteins generated a single 40 kDa protein species (M. M. R., H. Adkins, and J. A. T. Y.; unpublished data), demonstrating that the major proteins precipitated by SUB–rIgG were glycosylated forms of the cloned factor. Flow cytometry demonstrated that human 293 cells transfected with the 7.6-2 cDNA clone specifically bound SUB–rIgG (data not shown). The ability of the cloned factor to bind directly to ALV-B SU is an activity expected of a specific viral receptor; we therefore designated this protein CAR1 (cytopathic ALSV receptor).

CAR1 Is a Member of the TNFR Family

Several features identified CAR1 as a member of the TNFR family that includes TNFR1, TNFR2, Fas, p75^{NTR}, OX-40, CD40, CD27, and CD30 (reviewed by Beutler et al., 1994). The predicted extracellular region of CAR1 contains two TNFR-like cysteine-rich domains (CRDs) (Figure 4A). Both domains contain six highly conserved cysteines, which in TNFR1 are organized into three intradomain disulfide bonds (Banner et al., 1993). The second CRD of CAR1 contains two additional cysteines (residues 109 and 115; Figure 4A), as do the third CRDs of Fas (Itoh et al., 1991), TNFR2 (Smith et al., 1990), and CD40 (Stamenkovic et al., 1989). Additional conserved amino acid residues were identified in both CRDs of

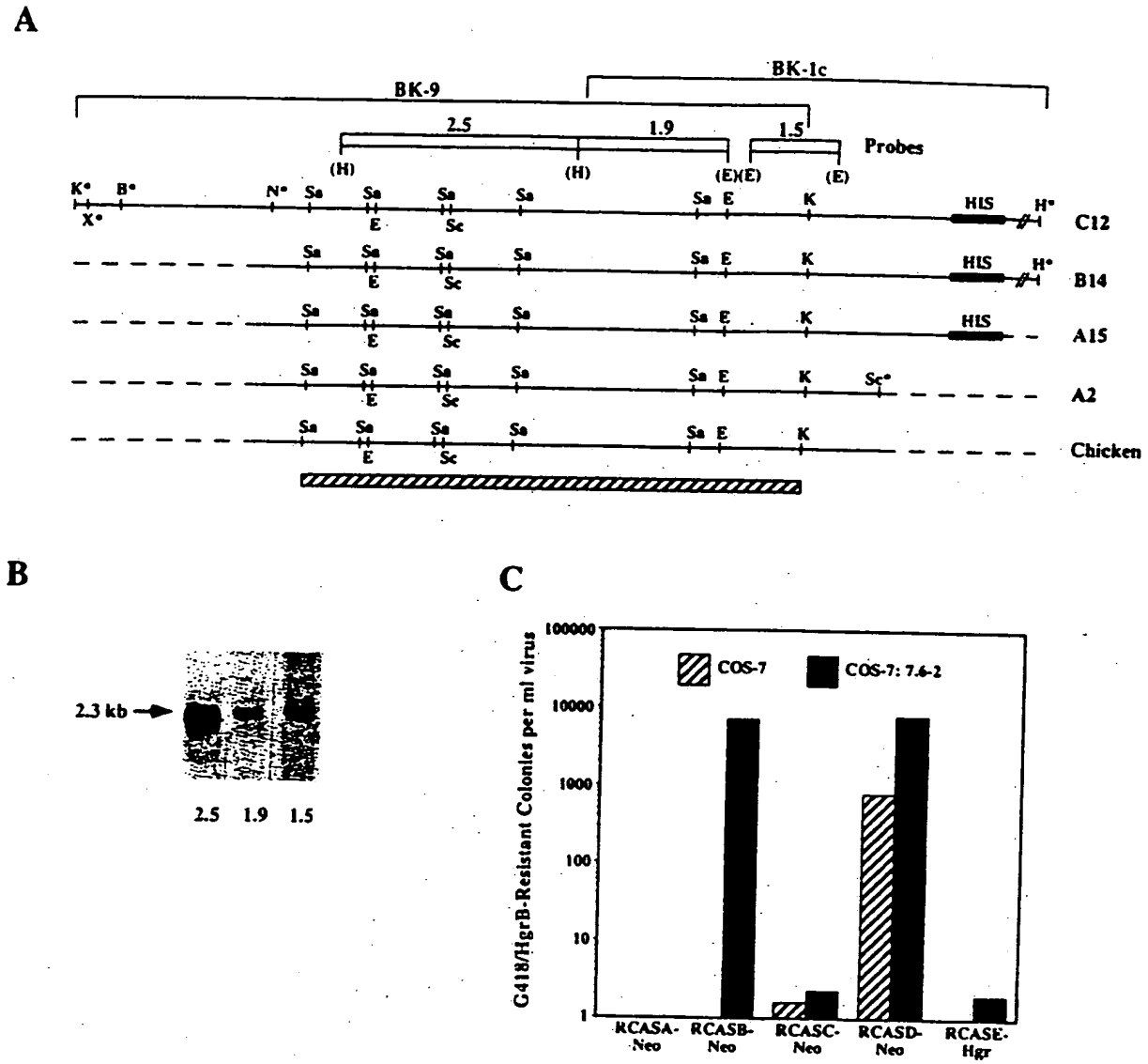

Figure 1. Isolation of a cDNA Clone Encoding an ALV-B Susceptibility Factor

(A) Restriction enzyme site map of a 7.5 kb chicken DNA locus shared between independent secondary transfectants (A2, A15, B14, and C12) that were susceptible to ALV-B. The 2.5 kb and 1.5 kb fragments (open boxes) were used as hybridization probes. The pMPHIS plasmid is indicated by a black box. The location of BK-9 and BK-1c clones is indicated. The shared 7.5 kb region of chicken genomic DNA is indicated by a striped box. Enzyme abbreviations: B, BglII; E, EcoRI; H, HindIII; N, NdeI; K, KpnI; Sa, SacI; Sc, ScaI; X, XbaI. Unique sites are indicated by asterisks. Sites in parentheses were used to generate probes but were not tested in every transfectant.

(B) Northern blot analysis of total RNA from chicken cells that were homozygous for *tv-b*^{a3} was performed using the 2.5 kb, 1.9 kb, and 1.5 kb fragments as hybridization probes.

(C) COS-7 cells transfected with the 7.6-2 cDNA clone were susceptible to ALV-B infection and showed an increased susceptibility to infection by mammal-tropic subgroup D viruses (Bova et al., 1988). The transfected cells were challenged with subgroup A, B, C, and D viruses conferring resistance to G418 and with a subgroup E virus conferring resistance to hygromycin B. The resultant numbers of G418 or hygromycin B-resistant colonies are indicated per ml virus.

CAR1, some of which in TNFR1 are required for proper folding of the structural core of these motifs (Banner et al., 1993; Figure 4A). Compared with the extracellular domains of other TNFR-like proteins, the regions of CAR1 with the most amino acid spacing differences (residues Thr-68 to Lys-76; Arg-81 to Gln-87; Cys-109 to Met-117; Pro-126 to Gln-129) correspond to two variable loop regions in the CRDs of TNFR1 (Banner et al., 1993).

The predicted cytoplasmic tail of CAR1 contained a region strikingly similar to the defined death domains of TNFR1 and Fas (Figure 4B), which are important for the ability of these receptors to induce apoptosis (Itoh and Nagata, 1993; Tartaglia et al., 1993). Similar death domains have been described in other apoptosis-inducing proteins including p75^{NTR} (Chapman, 1995), TRADD, FADD/MORT-1, and RIP (Figure 4B; reviewed by Cleveland and Ihle, 1995). The putative death domain of CAR1 was most highly related to the death domain of human TNFR1 (31% identity). Significantly, six residues in the death domain of TNFR1 shown to be critical for cell

A

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1     ATG CGC TCA GCT GCG CTC CGG TTG TGC CCC GTT CTA CTG CTG CTC TTC GCG GAG GTT CAG
1      M   R   S   A   A   L   R   L   C   P   V   L   L   L   L   F   A   E   V   Q
61    TTG GGA TCT GCT GCA GCA GTG AAG AAG AGG GCA GAC AGG TCA GAC CTC CAG AAG CCA GAC
21     L   G   S   A   A   A   V   K   K   R   A   D   R   S   D   L   Q   K   P   D
121   CTC TAC AGA AGG AAG TGT CCT ATG GGC ACC TAT GAG GCA AAT GAC TCC ATC CAG TGC CTC
41     L   Y   R   R   K   C   P   M   G   T   Y   E   A   N   D   S   I   Q   C   L
181   CCA AGT AAG AAA GAC GAG TAC ACC GAG TAT CCA AAT GAC TTT CCC AAG TGC CTG GGC TGC
61     P   S   K   K   D   E   Y   T   E   Y   P   N   D   F   P   K   C   L   G   C
241   CGG ACG TGT AGG GAA GAC CAG GTG GAG GTG AGT CCC TGC ATC CCC ACC AGG AAC ACG CAG
81     R   T   C   R   E   D   Q   V   E   V   S   P   C   I   P   T   R   N   T   Q
301   TGC GCT TGC AAG AAC GGC ACC TTC TGC TTA CCT GAC CAC CCC TGT GAG ATG TGC CAA AAG
101    C   A   C   K   N   G   T   F   C   L   P   D   H   P   C   E   M   C   Q   K
361   TGC CAG ACC GAG TGC CCC AAA GGA CAA GTG AGG TTA GCT CCG TGC ACG CAA CAC AGC GAC
121    C   Q   T   E   C   P   K   G   Q   V   R   L   A   P   C   T   Q   H   S   D
421   CTG CTG TGC GGT CCA CCC TTG GAA ATC TCC TCC AGC TCC TCC ACT TTA TGG ATC ATC ATC
141    L   L   C   G   P   P   L   E   I   S   S   S   S   S   T   L   W   I   I   I
481   ACC TTC ACC GTG CTG CTG GCT GTG ATC CTG GGG CTC GTG CTG GTG TTC TGG AAG AGG TGC
161    T   F   T   V   L   L   A   V   I   L   G   L   V   L   V   F   W   K   R   C
541   TCC TCC AGA CAC CAC GGT GCA GGG GAT GAT GGA GAG CTG AGC TGG AAG CCC AGC GCC GTG
181    S   S   R   H   H   G   A   G   D   D   G   E   L   S   W   K   P   S   A   V
601   GTG AAC AGA CTG TTG CAG CGG CTG GGG ATT CAG GAC AAC AGA TGC AAT GAG CAG ATC TAC
201    V   N   R   L   L   Q   R   L   G   I   Q   D   N   R   C   N   E   Q   I   Y
661   CAG AAC CAG CAG CAG CAG GAG CTG CTT TTC ACA GCG CAG GGC TCA GAG GTT CCC CAT GGT
221    Q   N   Q   Q   Q   Q   E   L   L   F   T   A   Q   G   S   E   V   P   H   G
721   GTG GAG ATG GAG GGG ACG GAA CGA AGA ACC CCA GAT CCC AAA GTG GAA ACC CAG AGG AAG
241    V   E   M   E   G   T   E   R   R   T   P   D   P   K   V   E   T   Q   R   K
781   CTG GTT CCA GTG CTA GGA GAG AAC CCC ATA GCC CTT TTG CAT CGC TCT TTC AAC ACC TTT
261    L   V   P   V   L   G   E   N   P   I   A   L   L   H   R   S   F   N   T   F
841   GTC GAC TAT GTG CCC TTC CCG GAA TGG AAG AGA TTT GGC CGA GCC CTC GAC CTG CAG GAA
281    V   D   Y   V   P   F   P   E   W   K   R   F   G   R   A   L   D   L   Q   E
901   AAC GAC CTT TAT CTG GCA GAG CAG CAC GAC AGG GTC TCA TGT GAG CCG TTC TAT CAG ATG
301    N   D   L   Y   L   A   E   Q   H   D   R   V   S   C   E   P   F   Y   Q   M
961   CTC AAC ACG TGG CTC AAC CAA CAG GGC AGC AAA GCC TCT GTG AAT ACG CTG CTG GAG ACC
321    L   N   T   W   L   N   Q   Q   G   S   K   A   S   V   N   T   L   L   E   T
1021  CTG CCC CGC ATC GGC CTC AGC GGC GTG GCA GAC ATA ATT GCA TCC GAA CTC ATT AGC AAG
341    L   P   R   I   G   L   S   G   V   A   D   I   I   A   S   E   L   I   S   K
1081  GGC TAT TTC CAG TAC GAG GTG AGC TGA
361    G   Y   F   Q   Y   E   V   S   •
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B

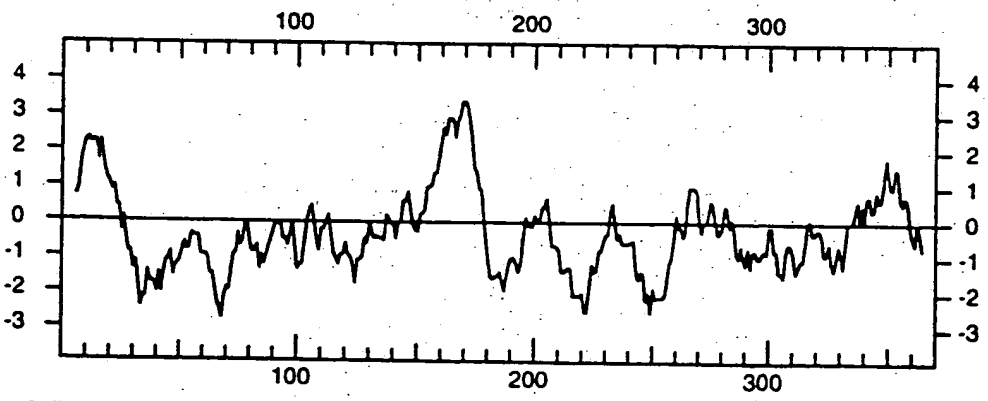

Figure 2. The 7.6-2 cDNA Clone Encodes a Type I Membrane Protein

(A) The long open reading frame contained within the 7.6-2 cDNA clone. The putative transmembrane domain is underlined, and two potential N-linked glycosylation sites (N-X-S/T) are boxed. The predicted leader peptidase cleavage site (von Heijne, 1986) is marked by an arrow.
(B) A hydropathy plot of the protein created using the algorithm of Kyte and Doolittle (1982).

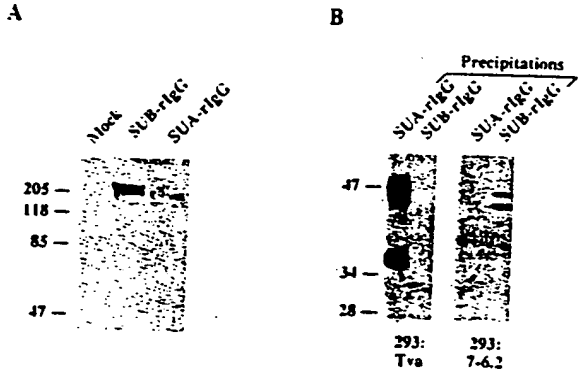

Figure 3. The Cloned Factor Bound Specifically to an ALV-B SU–Ig Fusion Protein

(A) Extracellular supernatants containing no immunoadhesin (mock), SUA–rIgG, or SUB–rIgG were subjected to electrophoresis under nonreducing conditions on a 7.5% polyacrylamide gel containing SDS and were immunoblotted using a horseradish peroxidase–coupled antibody specific for rabbit immunoglobulins.

(B) ^{35}S-labeled proteins from lysates of human 293 cells that had been transfected either with an expression vector encoding Tva (Zingler et al., 1995) or with the 7.6-2 cDNA clone were precipitated by the SU-immunoadhesins and protein A–Sepharose and analyzed by SDS–polyacrylamide gel electrophoresis. The sizes of the molecular mass markers are given in kDa.

killing (Tartaglia et al., 1993) appear to be absolutely conserved in this region of CAR1 (Figure 4B). Therefore, the cytoplasmic domain of CAR1 contains a region with many of the hallmarks of a functional death domain.

CAR1 Induces Apoptosis in Avian Cells

TNFR1 and Fas are known to signal apoptosis following receptor cross-linking upon binding to their cognate trimeric ligands or to receptor-specific antibodies (reviewed by Nagata and Golstein, 1995). Therefore, we decided to test whether the dimeric SUB–rIgG fusion protein that is able to bind CAR1 could elicit apoptosis. First, we determined if incubation with SUB–rIgG affected the viability of avian cells that expressed subgroup B viral receptors. CEFs (homozygous for the *tv-b*^{s2a} allele) that express subgroup B viral receptors and chicken cells (homozygous for the *tv-b*^{r} allele) that presumably do not express these receptors were used for these studies. These cells were incubated with increasing amounts of SUB–rIgG in the presence of a protein biosynthesis inhibitor, cycloheximide, which is routinely included in apoptosis assays (e.g., Laster et al., 1988). Following a 6 day incubation period with SUB–rIgG, the cell population that expressed subgroup B viral receptors showed a marked reduction in the numbers of adherent cells (Figure 5A). The magnitude of this effect was dependent upon the concentration of SUB–rIgG added (Figure 5A) and required cycloheximide. In contrast, the SUB–rIgG protein did not affect the numbers of adherent cells observed with the cell population not expressing functional subgroup B viral receptors (Figure 5A).

The nonadherent cells that resulted from incubations with SUB–rIgG and cycloheximide contained nucleosomal genomic DNA ladders, indicating that they had undergone apoptosis (data not shown). To obtain further evidence that SUB–rIgG induced apoptosis in a manner that was dependent on expression of the subgroup B viral receptor, this immunoadhesin was incubated with CEFs (homozygous for the *tv-b*^{s2} allele) that were either uninfected or chronically infected by the subgroup B virus RCASH-B. Cells chronically infected by this virus have presumably survived any virus-induced cell death (Weller et al., 1980; Weller and Temin, 1981) and are resistant to viral superinfection owing to functional down-regulation of the subgroup B viral receptor (Weiss, 1993). For control purposes, these experiments were also performed with CEFs chronically infected by the subgroup A virus RCASH-A and with the SUA–rIgG protein, which was not expected to induce apoptosis. To quantitate the degree of apoptosis induced in each cell population, we used an ELISA–based assay to measure the level of cytoplasmic nucleosomal DNA fragments. The uninfected cells and the subgroup A virus–infected cells showed significantly increased apoptosis in the presence of SUB–rIgG and cycloheximide, compared with incubation with SUA–rIgG and cycloheximide (Figure 5B). However, cells chronically infected with the subgroup B virus did not die preferentially when incubated with SUB–rIgG, presumably because subgroup B viral receptors had been functionally down-regulated (Weiss, 1993). These data provided further evidence that the subgroup B viral receptor was important for inducing cell death.

To obtain direct evidence that the subgroup B viral receptor CAR1 can induce apoptosis, a cloned line of transfected quail QT6 cells stably expressing this factor was incubated with SUB–rIgG protein that had been purified to 80%–90% homogeneity using a protein A column (Zingler and Young, 1996). CAR1 expression was confirmed in these cells by immunoblot analysis (data not shown). As expected, these cells were infected by RCASH-B in contrast to wild-type QT6 cells (Figure 5C), which are normally resistant to subgroup B viral infection (Weiss, 1993). Only the QT6 cells expressing CAR1 were induced to undergo apoptosis in response to SUB–rIgG (Figure 5D). Similar results were obtained with a second independent clone of QT6 cells that expressed CAR1 (data not shown). These results demonstrate that SUB–rIgG/CAR1 interactions can lead to the death of avian cells.

Discussion

A gene was cloned encoding a TNFR–related protein, CAR1, which permitted specific infection of mammalian cells by ALV-B and ALV-D. This protein bound selectively to a subgroup B ALV SU–Ig fusion protein (SUB–rIgG). Taken together, these observations indicate that CAR1 most likely mediates viral entry by serving as a specific receptor for viral subgroups B and D. CAR1 is apparently not related to any other known retroviral receptor; these include Tva, a receptor for another avian viral subgroup (ALV-A), which is a member of the low density lipoprotein receptor family; CD4, a member of the immunoglobulin protein superfamily; and transporter proteins that contain multiple membrane-spanning regions (Weiss and Tailor, 1995).

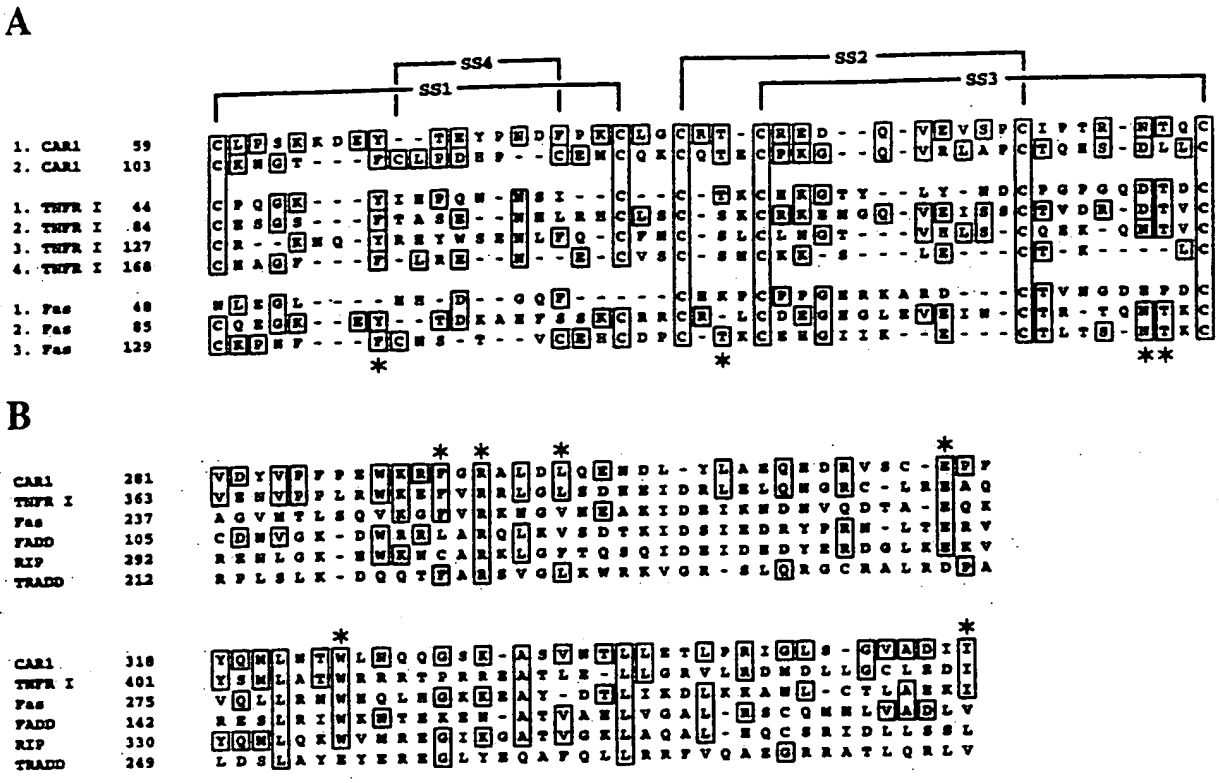

Figure 4. CAR1 Is a Member of the TNFR Superfamily

(A) Alignment of the extracellular CRDs of CAR1 with the corresponding regions of the human TNFR1 (Loetscher et al., 1990; Schall et al., 1990) and human Fas (Itoh et al., 1991). Boxed amino acids indicate amino acids identical to CAR1. A set of three disulfide bonds in TNFR1 (Banner et al., 1993) is indicated as SS1–SS3. SS4 represents a putative disulfide bond found in CRDs of some TNFR-related proteins including the second CRD of CAR1. Asterisks indicate amino acid residues which, in addition to the cysteines, stabilize the conformation of TNFR1 CRDs.

(B) CAR1 contains a putative death domain. Alignment of a cytoplasmic region of CAR1 with the death domains of other proteins. Boxed amino acids indicate amino acids identical to CAR1, and asterisks indicate residues of TNFR1 that are essential for cell killing (Tartaglia et al., 1993).

CAR1 contains two extracellular CRDs similar to those found in the TNFR family. Based on the known structure of the four domains of TNFR1 (Banner et al., 1993), the six cysteines in the first CRD of CAR1 are predicted to form three intradomain disulfide bonds (Figure 4A). The eight cysteines in the second CRD of CAR1 are predicted to have the same pattern of disulfide bonds with an additional putative bond formed between Cys-109 and Cys-115 (Figure 4A). It is intriguing that the ALV-A interaction site of Tva is contained within a low density lipoprotein receptor–related motif that is similar in size to TNFR–like CRDs and is predicted to contain three intradomain disulfide bonds (Bélanger et al., 1995; Daly et al., 1995; Zingler et al., 1995). This similarity suggests that viral interaction sites of Tva and CAR1 might have common features.

The CRDs of TNFR1 are known to adopt similar core structures (Banner et al., 1993), and residues important for the stability of this structure have been identified. These include highly conserved Asp/Asn–Thr residues (located before the last cysteine residue), which are hydrogen-bonded to a conserved serine or threonine residue located after the third cysteine (Figure 4A). Also, a conserved aromatic residue (Tyr or Phe) located approximately five residues after the first cysteine provides further stability by interacting both with the second disulfide bridge and the Asp/Asn:Ser hydrogen bond bridge (Banner et al., 1993). A number of these important amino acid residues are also found in CAR1 (Tyr-67, Thr-82, Asn-98, Thr-99, Phe-108, Thr-123, and Asp-140), suggesting that both CRDs of the ALV receptor are structurally related to those of TNFR1 (Figure 4A).

The presence of a cytoplasmic region in CAR1 similar to the death domain of Fas and TNFR1 (Figure 4B) led to experiments demonstrating that this ALV receptor could induce apoptosis in avian cells. CEFs that expressed subgroup B viral receptors underwent apoptosis in response to the ALV-B SU immunoadhesin, whereas cells that lacked these receptors did not. In addition, preinfection of CEFs by ALV-B led to protection against apoptosis induced by this immunoadhesin. Presumably, this protection is due to subgroup B viral receptor down-regulation in these cells (Weiss, 1993), although subgroup B virus infection might have selected for a cell population resistant to SUB–rIg-induced apoptosis. Furthermore, the subgroup B SU immunoadhesin caused apoptosis in quail QT6 cells that stably expressed CAR1, but not in wild-type QT6 cells. These data demonstrate that cytopathic ALV Env–receptor interactions can lead to cell death and might

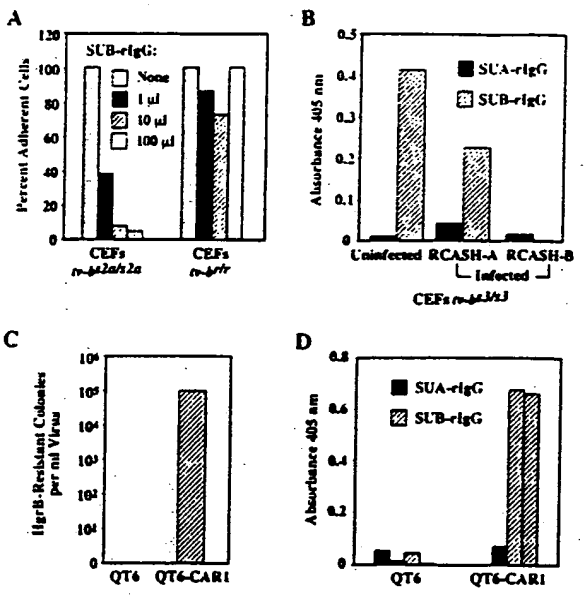

Figure 5. CAR1-Mediated Apoptosis

(A) CEFs susceptible to ALV-B infection are preferentially killed in response to SUB–rIgG. A representative experiment in which approximately 2 × 10^5 CEFs that either expressed (*tv-b*$^{s3/s13}$) or lacked (*tv-b*$^{r/r}$) functional subgroup B viral receptors were incubated with increasing amounts (1, 10, and 100 μl) of extracellular supernatants containing SUB–rIgG (Figure 3A) in the presence of 7.5 μg/ml of cycloheximide. The numbers of adherent cells were counted after 6 days and corrected relative to the numbers of cells remaining after incubation with cycloheximide alone.

(B) CEFs chronically infected by ALV-B are protected from SUB–rIgG-induced apoptosis. A representative experiment in which extracellular supernatants containing SUA–rIgG or SUB–rIgG (Figure 3A) were incubated with 2 × 10^4 CEFs that express subgroup B viral receptors (*tv-b*$^{s3/s3}$) and that were either uninfected or chronically infected with the subgroup A virus RCASH-A or with the subgroup B virus RCASH-B. These experiments were performed for 2 days in the presence of 10 μg/ml of cycloheximide. Cells were assayed for evidence of apoptosis using an ELISA–based assay that measures photometrically the amount of cytoplasmic histone-associated DNA fragments. The apparent reduction in the level of SUB–rIgG-induced apoptosis obtained with RCASH-A–infected cells probably reflects their observed reduced plating efficiency in these experiments.

(C) A cloned line of QT6 cells stably expressing CAR1 was susceptible to infection by the RCASH-B virus. The number of hygromycin B-resistant colonies that resulted from viral infection is shown.

(D) Quail QT6 cells that expressed CAR1 underwent apoptosis in response to SUB–rIgG. Approximately 2 × 10^4 QT6 cells or QT6 cells expressing CAR1 were incubated with 16 ng of either purified SUB–rIgG or purified SUA–rIgG in the presence of 2.5 μg/ml of cycloheximide. The apoptosis assays were performed as described in (B) above, and the results were from two independent experiments.

explain why cells killed by these viruses contain "ladders" of degraded genomic DNA fragments (Weller and Temin, 1981) that presumably result from apoptosis (e.g., Itoh et al., 1991).

It seems likely that CAR1 triggers cell death in a manner similar to Fas and TNFR1; cross-linking of these receptors by their trimeric ligands leads to aggregation of their death domains, resulting in apoptosis (Itoh and Nagata, 1993; Nagata and Golstein, 1995; Smith et al., 1994; Tartaglia et al., 1993; Watanabe-Fukunaga et al., 1992). Cytoplasmic proteins have been identified that bind directly to the wild-type death domain of either Fas or TNFR1 and induce or potentiate apoptosis. These include TRADD (Hsu et al., 1995), FADD/MORT1 (Chinnaiyan et al., 1995; Boldin et al., 1995), and RIP (Stanger et al., 1995). It seems reasonable to expect that similar types of proteins interact with the death domain of CAR1 and may be involved in signaling apoptosis by this cellular receptor.

The fact that CAR1 permits specific infection of mammalian cells by ALV subgroups B and D but not E indicates that it is most likely the product of *tv-b*^{s3}, the chicken locus predicted to encode cellular receptors for these two ALV subgroups (Weiss, 1993). If future gene-mapping studies confirm this locus designation, it will be important to isolate and characterize any products of tv-b^r (which do not allow infection by cytopathic subgroup B and D viruses; Weiss, 1993). Any differences between these proteins and CAR1 might help identify the viral interaction determinants of the receptor. Comparison of *tv-b* alleles that encode receptors for noncytopathic subgroup E viruses (Weiss, 1993) with CAR1 might provide crucial insights into the mechanisms of cell killing associated with ALV-B and ALV-D.

Viruses have evolved various strategies to evade host-cell killing by encoding inhibitors of apoptosis (reviewed by Thompson, 1995). These include the Bcl-2 homologs BHRF1 and LMW5-HL of Epstein-Barr virus and African swine fever virus, respectively (Neilan et al., 1993; Henderson et al., 1993); the cowpox virus crmA protein, which is a specific inhibitor of IL-1β–converting enzyme (Ray et al., 1992); and the myxoma virus T2 protein (a soluble TNFR–like protein), which can protect cells from lysis by TNF (Schreiber and McFadden, 1994). Given that other viruses have devised strategies to evade apoptosis, it seems counterintuitive that cytopathic subgroups of ALV would utilize a cellular receptor that could induce apoptosis in the host cell. A hypothesis that might explain this apparent paradox is that functional down-regulation of the subgroup B viral receptor that follows ALV-B infection (Weiss, 1993) might provide a selective advantage for the virus. For example, given that Fas is important for the elimination of virus-infected cells by cytotoxic T lymphocytes (Lowin et al., 1994; reviewed by Nagata and Golstein, 1995) and that TNFR1 has been shown to be necessary for clearing Listeria monocytogenes infection (Pfeffer et al., 1993), CAR1 might also be involved in immune clearance of virus-infected cells. If so, functional down-regulation of CAR1 following infection by subgroup B or D ALV might protect the host cell from apoptosis by this pathway. Consistent with this hypothesis, cells infected by ALV-B were protected from apoptosis induced by SUB–rIgG (Figure 5B). This would be a novel mechanism for evading apoptotic elimination of infected cells.

In conclusion, our results indicate that subgroups B and D ALV Env–CAR1 interactions may contribute to the virus-associated CPE by inducing apoptosis. This model would explain why the determinants of subgroup B viral receptor usage on ALV-B SU Env proteins appear to be the same as those responsible for the cytopathic effect (Domer and Coffin, 1986). Presumably, cell-surface expression of functional subgroup B viral receptors is important for the viral CPE as well as for the associated

viral superinfection. This would explain why chronically infected cells, which have functionally down-regulated subgroup B viral receptors, are resistant both to viral superinfection and to cell killing (Weller et al., 1980; Weller and Temin, 1981). It is possible that viral superinfection contributes to ALV-induced cell killing (Weller et al., 1980; Weller and Temin, 1981), although multiple rounds of viral infection may simply be a consequence of cell death. Env–receptor interactions have also been implicated in HIV-1–induced CPE (Lu et al., 1994; Cao et al., 1996; Banda et al., 1992), although the exact details of the mechanisms of cell killing are probably different because of the absence of a death domain in the viral binding receptor, CD4. It remains to be seen whether other proteins containing death domains are used as cellular receptors by different cytopathic retroviruses, including ALV-F (Weller et al., 1980; Weller and Temin, 1981), spleen necrosis virus (Keshet and Temin, 1979), and subgroup C feline leukemia viruses (Riedel, 1988).

Experimental Procedures

Cells and Viruses

Mouse NIH 3T3 cells, Quail QT6 cells, Monkey COS-7 cells, Human 293 cells, and primary CEFs were grown and selected as previously described (Young et al., 1993; Bates et al., 1993; Connolly et al., 1994; Zingler and Young, 1996). Chicken fibroblasts were obtained from the Avian Disease and Oncology Laboratory, USDA Poultry Laboratories (East Lansing, Michigan). Cells homozygous for *tv-bs*^{r} were derived from line 0 chickens, those homozygous for *tv-b*^{r} were derived from a cross between line 100B and line 7 chickens, and those homozygous for *tv-b*^{a2a} were derived from line 15B1 chickens. The subgroup B–specific RCASH-B virus containing the hygromycin B phosphotransferase gene driven by the HSV thymidine kinase (tk) promoter was described previously (Young et al., 1993). The subgroup E–specific RCASH-E virus was derived from RCASH-B by replacing a 1.1 kb KpnI–SalI *env* fragment with that from the RAV-0 viral strain. The subgroup A–specific RCASA-neo, subgroup B–specific RCASB-Neo, and subgroup D–specific RCASD-neo viruses were generated from previously described proviral DNAs (Connolly et al., 1994) by digestion with ClaI followed by end-filling with Klenow polymerase to generate blunt-ended cloning sites. An end-filled 1.2 kb XhoI HincII fragment containing the HSV-tk promoter and the neomycin phosphotransferase gene from plasmid pMCI.neo PA– (gift from M. Feinberg) was introduced into the blunt-ended cloning site of each viral vector. The subgroup C–specific RCASC-neo virus was derived by first subcloning a similar XhoI–HindIII fragment from plasmid pMCI.neo PA– into plasmid SACla12Nco (Hughes et al., 1987). A ClaI–ClaI fragment containing these DNA sequences was then used to replace the hygromycin B phosphotransferase gene of the RCASH-C virus (Connolly et al., 1994).

DNA Transfections, Radioactively Labeled Probes, and Nucleic Acid Hybridizations

All DNA transfections were performed by the calcium phosphate precipitation method (Wigler et al., 1977). The radioactively labeled probes were prepared by the random priming method (Feinberg and Vogelstein, 1984). DNA and RNA samples that were transferred to a Genescreen plus (DuPont) membrane were hybridized with probes at 65°C using standard conditions (Church and Gilbert, 1984) unless otherwise indicated. The membranes were exposed for autoradiography to Kodak XAR-5 film with intensifying screens at −80°C.

Genomic DNA Transfections and Infection by Subgroup B ALV Vectors

A total of 6 × 10^{6} mouse BALB/3T3 cells were cotransfected with 120 μg of genomic DNA (sheared to an average size of approximately 20 kb) prepared from CEFs homozygous for *tv-b*^{a1} and with 12 μg of pMPHis plasmid DNA encoding histidinol dehydrogenase (Young et al., 1993). After 48 hr, the cells were selected in histidine-free medium containing 1 mM histidinol. Cells derived from these colonies were challenged over a 3 day period with a total of 6 × 10^{6} infectious units of RCASH-B virus and 1 day later were selected in medium containing 300 μg/ml of hygromycin B. Cells derived from the resultant hygromycin B–resistant colonies were plated out at 20% confluency in 6-well plates, challenged with 10^{4} infectious units of RCASB-neo virus, and 1 day later were selected in medium containing 300 μg/ml of G418.

A total of 1.4 × 10^{7} mouse BALB/3T3 cells were transfected with 200 μg of genomic DNA (from an aliquot of primary transfectant 11B cells that were infected only by RCASH-B) and with 20 μg of plasmid pPur encoding puromycin-N-acetyl-transferase (Clontech). After 2 days, secondary transfectants were selected in media containing 2 μg/ml of puromycin. Cells derived from the puromycin-resistant colonies were challenged with a total of 2 × 10^{7} infectious units of the RCASB-neo virus and 1 day later were selected in medium containing 300 μg/ml of G418. Cells derived from the resultant G418-resistant colonies were plated at 20% confluency in 6-well plates, challenged with 5 × 10^{3} infectious units of subgroup B–specific RCASH-B virus, and 1 day later were selected in medium containing 300 μg/ml of hygromycin B.

Genomic DNA Clones

HindIII–HindIII restriction fragments approximately 6.5–8 kb in size and KpnI–KpnI restriction fragments approximately 8–10 kb in size (derived from C12 secondary transfectant genomic DNA) were size-selected by agarose gel electrophoresis and introduced into the λZAP vector (Stratagene), generating libraries of approximately 300,000 and 150,000 recombinant clones, respectively. The library of HindIII fragments was subjected to three rounds of screening using a 2 kb PvuII–PvuII fragment from the pMPHIS plasmid as a hybridization probe. Following a standard phagemid excision protocol (Stratagene), the BK-1c clone was isolated. The library of KpnI fragments was subjected to three rounds of screening by hybridization with the 1.9 kb EcoRI–HindIII and 1.5 kb EcoRI–EcoRI fragments from clone BK-1c. This led to the isolation of the genomic DNA clone BK-9.

Northern Blot Analysis and cDNA Cloning

Samples of approximately 10 μg of total RNA prepared from CEFs homozygous for *tv-b*^{a1} were subjected to 1% agarose gel electrophoresis in the presence of formaldehyde (Ausubel et al., 1992), transferred to a nylon membrane, and subjected to hybridization with radioactively labeled probes.

Approximately 5 μg of polyadenylated mRNA from these CEFs was reverse-transcribed to generate cDNA using a commercially available kit (ZAP Express cDNA synthesis kit; Stratagene) and introduced into the λZAP Express vector (Stratagene). Approximately 225,000 recombinant clones were transferred to nylon membranes and subjected to three rounds of screening by hybridization with the radioactively labeled 2.5 kb, 1.9 kb, and 1.5 kb probes derived from the BK-9 and BK-1c genomic clones. Following hybridization, the membranes were washed at 56°C with 2 × SSC prior to autoradiography. Using a standard phagemid excision protocol (Stratagene), plasmid pBK7.6-2 was isolated, and the cDNA clone was subsequently sequenced by the chain termination method (Sanger et al., 1977).

Construction, Purification, and Analysis of SU-Immunoadhesins

The SUB–rIgG protein comprised amino acids 1–344 of the subgroup B SU protein derived from the RCASH-B virus (Connolly et al., 1994) fused in-frame to the Fc region (amino acids 96–323) of a rabbit IgG heavy chain (SwissProt accession number P01870). The SUA–rIgG protein comprised amino acids 1–338 of the subgroup A ALV SU protein derived from the RCASH-A virus (Young et al., 1993) fused to the same immunoglobulin sequences. The corresponding DNA sequences were fused together following the introduction of in-frame BamHI sites in each construct by standard polymerase chain reaction–based mutagenesis protocols (Ausubel et al., 1992). The resultant *SUA–rIgG* and *SUB–rIgG* genes were introduced into the

pCB6 mammalian expression vector (Bates et al., 1993). These proteins were released in the extracellular supernatants of transiently transfected human 293 cells and purified as described elsewhere (Zingler and Young, 1996).

Samples of 25 μl transfected cell supernatants were subjected to 7.5% SDS–polyacrylamide gel electrophoresis under nonreducing conditions, and the SU-immunoadhesins were detected by immunoblotting with a horseradish peroxidase–coupled donkey antibody specific for rabbit immunoglobulins and by enhanced chemiluminescence (Amersham) as described elsewhere (Zingler and Young, 1996). The SU-immunoadhesins were purified using a protein A column as described elsewhere (Zingler and Young, 1996).

Tva and CAR1 Binding to Immunoadhesins

Human 293 cells plated at approximately 20% confluence on 100 mm tissue culture plates were transfected with 15 μg of a plasmid encoding Tva (Zingler et al., 1995), 15 μg of plasmid pBK7.6-2 encoding CAR1, or no DNA. The transfected cells were metabolically labeled for 2 hr with Dulbecco's modified Eagle's medium without cysteine and methionine containing 1% dialyzed fetal calf serum and 100 μCi/ml ^{35}S-cysteine (ICN Chemicals). Cell lysates were prepared in a lysis buffer containing NP-40 as described previously (Bélanger et al., 1995). Aliquots (one-tenth) of each sample were then incubated at 4°C for 45 min with 10 μl of protein A–Sepharose beads (Sigma) and 30 μl of Sepharose CL-4B (prebound to 1 ml of extracellular supernatants containing SUA–rIgG or SUB–rIgG for 45 min). The bound proteins were collected by centrifugation, and aliquots of these samples were subjected to electrophoresis on a 10% SDS–polyacrylamide gel under reducing conditions. The gel was dried and exposed to Kodak XAR-5 film at room temperature.

Infection of Transfected COS-7 Cells with Different Subgroups of ALV

COS-7 cells were transfected with 20 μg of 7.6-2 plasmid pBK7.6-2 Amp (derived from pBK7.6-2 by replacement of the neomycin phosphotransferase gene with the ampicillin resistance gene of pBluescript KS− [Stratagene]). Transfected cells were challenged with serial 10-fold dilutions of the virus stocks. The titers of the undiluted stocks of RCASA-neo, RCASB-neo, RCASC-neo, and RCASD-neo viruses were approximately 10^5 infectious units/ml as determined by limiting dilution infection of CEFs. The titer of the undiluted stock of RCASH-E virus was approximately 10^3 infectious units/ml as determined by limiting dilution infection of turkey embryo fibroblasts. Between 24–36 hr after infection, the cells were selected in media containing 300 μg/ml of either hygromycin B or G418. Drug-resistant colonies were counted 11–14 days after infection and the numbers corrected per ml of undiluted virus.

Cell-Killing Assays

CEFs chronically infected with either RCASH-A or RCASH-B were generated by infecting 5×10^5 cells with approximately 10^5 infectious units of virus. Following two cell passages to allow viral spread, infected cells were selected in medium containing 80 μg/ml of hygromycin B. Cell-killing experiments were performed with uninfected and infected CEFs derived from the same embryo that had been passaged in culture for the same period of time (13 days). QT6 cells expressing CAR1 were generated by transfection with 15 μg of plasmid pBK7.6-2 followed by selection in medium containing 300 μg/ml of G418. Individual clones were tested for CAR1 expression by immunoblot analysis that employed SUB–rIgG and a horseradish peroxidase–coupled antibody specific for rabbit immunoglobulins. The cell-killing experiments were performed using the Cell Death Detection Plus™ ELISA kit (Boehringer Mannheim), in which cytoplasmic nucleosomes are purified by a histone-specific antibody and detected photometrically.

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nant magnetic carrier in the sheeted dike basalts, did not form simply by oxidation-exsolution, as has commonly been assumed (6, 12, 26). The natural remanent magnetization (NRM) of the sheeted-dike basalts was presumably acquired by the single-domain Ti-bearing magnetite (the host after exsolution) during initial cooling. This was a thermoremanent magnetization (TRM) and was then modified by chemical remanent magnetization of recrystallized end-member magnetite during hydrothermal alteration near the spreading center. The initial cooling and hydrothermal alteration appear to have taken place soon after the intrusion of basalts; therefore, the NRM reflects the original geomagnetic field direction (6).

The thickness of the source layers responsible for the sea-floor magnetic anomalies has long been debated and has been estimated as extending from the uppermost 500 to 1000 m (pillow basalts, layer 2A) of the oceanic crust to depths of ~8 km (essentially the entire oceanic crust) (3, 7, 27, 28). The results of studies of magnetic properties of sheeted dike basalts recovered from DSDP drill holes suggest that the sheeted dike complex (layer 2B) contributes significantly to sea-floor magnetic anomalies (6, 26, 29). However, magnetic data from ocean gabbros indicate that the linear magnetic anomalies originated partly in the gabbro layer (layer 3) (29–31). We have shown that single-domain, end-member magnetite, an efficient and stable carrier of TRM, is responsible for the magnetic properties in the upper levels (depths of ~630 m to at least ~1125 m within the igneous basement) of the sheeted dike complex at site 504B. The resultant NRM intensity of the sheeted dike basalts is on the same order as that of the pillow basalts at site 504B (6, 8, 12). We therefore conclude that the upper sheeted dike basalts from DSDP hole 504B are a significant source of sea-floor magnetic anomalies.

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14. Oxidation-exsolution is defined as oxidation reactions with oxygen partitioning into titanomagnetite but giving rise to well-oriented lamellar textures that are commonly caused by exsolution.
15. A Philips CM-12 scanning transmission electron microscope equipped with a Kevex (Chesham, United Kingdom) quantum detector was used. The methods for specimen preparation and STEM quantitative chemical analyses are described in (16).
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Induction of Apoptosis by the Low-Affinity NGF Receptor

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Nerve growth factor (NGF) binding to cellular receptors is required for the survival of some neural cells. In contrast to Trk A, the high-affinity NGF receptor that transduces NGF signals for survival and differentiation, the function of the low-affinity NGF receptor, $p75^{NGFR}$, remains uncertain. Expression of $p75^{NGFR}$ induced neural cell death constitutively when $p75^{NGFR}$ was unbound; binding by NGF or monoclonal antibody, however, inhibited cell death induced by $p75^{NGFR}$. Thus, expression of $p75^{NGFR}$ may explain the dependence of some neural cells on NGF for survival. These findings also suggest that $p75^{NGFR}$ has some functional similarities to other members of a superfamily of receptors that include tumor necrosis factor receptors, Fas (Apo-1), and CD40.

Growth factors such as NGF enhance the survival of cells displaying the appropriate receptors. The effects of NGF are mediated at least in part by Trk A, the high-affinity NGF receptor, which is a tyrosine kinase (1). The low-affinity NGF receptor, $p75^{NGFR}$, is a receptor of incompletely characterized function: $p75^{NGFR}$ has been shown to increase the affinity of Trk A for NGF (1) and to enhance the specificity of the Trk family of receptors for neurotrophins (2). $p75^{NGFR}$ has some sequence similarity to the tumor necrosis factor receptors [TNFR I (3) and TNFR II (4)], the human cell surface antigen Fas (Apo-1) (5), and the B cell antigen CD40 (6), all of which mediate cell death. In the case of TNFR I and Fas, binding of the receptor by ligand or antibody initiates cell death. In the case of CD40, however, binding by monoclonal antibody (mAb) or ligand inhibits cell death (6). Thus, some cells expressing CD40 are dependent on ligand or mAb binding for survival. Because of structural and functional analogies between the CD40 and $p75^{NGFR}$ systems, the possibility that $p75^{NGFR}$ serves as a constitutive cell death–promoting molecule that is inhibited by NGF binding was evaluated.

We expressed $p75^{NGFR}$ in temperature-

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Fig. 1. Expression of rat $p75^{NGFR}$ by transfected conditionally immortalized neural cells (*28*). (A) Northern blot demonstrating lack of Trk A and $p75^{NGFR}$ expression by CSM 14.1 cells. $p75^{NGFR}$ expression was demonstrated after transfection by pBabe-puro-$p75^{NGFR}$. Lane 1, CSM 14.1 transfected with pBabe-puro-$p75^{NGFR}$ and grown in serum-containing medium without NGF; lane 2, CSM 14.1 transfected with pBabe-puro-$p75^{NGFR}$ and grown in medium with serum and NGF (2 nM); lane 3, CSM 14.1 transfected with pBabe-puro and grown in serum-containing medium without NGF; lane 4, CSM 14.1 transfected with pBabe-puro and grown in medium with serum and NGF (2 nM); and lane 5, PC12 control. Note that the endogenous transcript in PC12 cells [3.7 kb (*29*)] is shorter than the transcript in the pBabe-puro-$p75^{NGFR}$–transfected cells (predicted to be 4.3 kb), and that treatment of the CSM 14.1 cells with NGF did not result in $p75^{NGFR}$ expression (lanes 2 and 4). Lanes 1 through 4 contained 25 μg of total RNA; lane 5 contained 10 μg of total RNA. (B) Immunocytochemistry of CSM 14.1 cells transfected with pBabe-puro-$p75^{NGFR}$ (85 ± 11% of cells expressed $p75^{NGFR}$). (C) Immunocytochemistry of CSM 14.1 cells transfected with pBabe-puro (0.6 ± 0.5% of the cells expressed $p75^{NGFR}$). Magnification, ×400.

sensitive immortalized neural cells (*7*) by means of a retroviral vector, pBabe-puro-$p75^{NGFR}$ (*8*) (Fig. 1). Control cells transfected with pBabe-puro expressed neither $p75^{NGFR}$ nor Trk A (Fig. 1). In cells cultured in medium containing serum, expression of $p75^{NGFR}$ had no effect on cell death, but when serum was withdrawn to induce apoptosis (*9*), expression of $p75^{NGFR}$ led to an increase in neural cell death (Fig. 2). However, if NGF (5 nM) was added, not only was the negative effect on cell survival suppressed, but the cells had a death rate less than that of control cells transfected with the identical vector lacking the $p75^{NGFR}$ sequence (Fig. 2). Binding of $p75^{NGFR}$ by a mAb also suppressed the enhancement of neural cell death by $p75^{NGFR}$, but led to less improvement of cell survival than did NGF (Fig. 2). Addition of a control mAb did not affect cell survival (Fig. 2). Neither NGF nor mAb affected survival of the control cells (Fig. 2).

We demonstrated that the type of cell death induced by $p75^{NGFR}$ was apoptotic by expressing $p75^{NGFR}$ in the R2 cell line, a conditionally immortalized cerebellar neural line (*10*) that, in the absence of $p75^{NGFR}$ expression, does not undergo apoptosis in serum-free medium. As shown in Fig. 3, expression of $p75^{NGFR}$ by the R2 cell line led to virtually complete cell death in serum-free medium, with the nuclear fragmentation, chromatin condensation, and homogeneous nuclear staining that are characteristic of apoptosis but not necrosis (*11*). Control R2 transfectants survived well in serum-free medium (Fig. 3).

It was possible that the mediation of neural cell death by $p75^{NGFR}$ might have been a result of the vector-driven expression of $p75^{NGFR}$ in neural cells that do not express endogenous $p75^{NGFR}$. Therefore, PC12 pheochromocytoma cells, which express $p75^{NGFR}$ (Fig. 1) and undergo apoptotic cell death after serum withdrawal

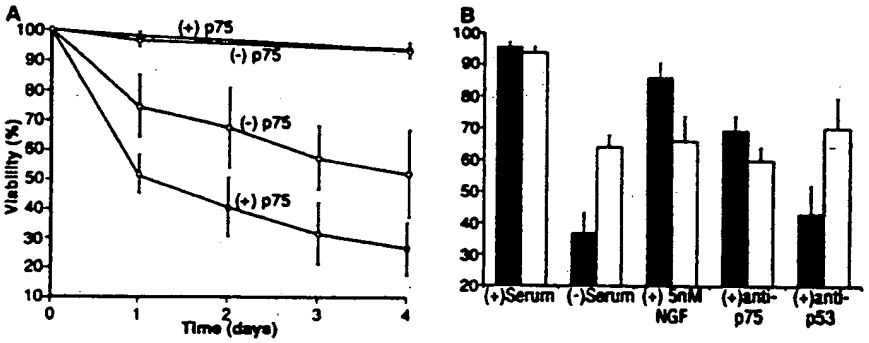

Fig. 2. Enhancement of neural cell death in cells expressing $p75^{NGFR}$. CSM 14.1 cells (*7*) were grown in DMEM with FBS (10%) at 34°C and then switched to the restrictive temperature of 39°C for 36 hours. Cell death was then induced by replacement of the medium with serum-free DMEM [either alone or in combination with NGF (5 nM)], and cell viability was determined each day for 4 days. Viable cells were identified by trypan blue exclusion and by propidium iodide fluorescence. Differences between cells expressing $p75^{NGFR}$ and control cells were highly statistically significant ($P = 0.0001$ by two-way analysis of variance, $n = 5$, from four different stable transfections of each plasmid). Error bars represent standard deviations. (A) Effect of serum-free medium on viability of cells expressing $p75^{NGFR}$ in comparison to control cells. Squares, cell transfected with pBabe-puro-$p75^{NGFR}$; circles, cells transfected with pBabe-puro; triangles, cells transfected with pBabe-puro-$p75^{NGFR}$, grown in medium with 10% serum; diamonds, cells transfected with pBabe-puro, grown in medium with 10% serum. (B) Effect of NGF (5 nM) and monoclonal antibodies (10 μg/ml) on cells expressing $p75^{NGFR}$ (closed bars) and control cells (open bars). Control mAb was directed against human p53 (anti-p53) (10 μg/ml) (Pharmingen). Each pair showed a highly significant difference ($P < 0.01$ by paired t test, $n = 3$), except the mAb to $p75^{NGFR}$ (anti-p75) ($P < 0.05$) and the controls (no significant difference).

(*12*), were studied. In the presence of mAb binding to $p75^{NGFR}$ (10 μg/ml), the number of cells undergoing cell death after serum withdrawal for 3 days was decreased from 78 ± 8% to 13 ± 4% ($P < 0.01$ by paired t test, $n = 3$), whereas the same concentration of control mAb did not affect cell survival. Furthermore, mutant PC12 cells lacking expression of $p75^{NGFR}$ (*13*) underwent very little cell death in serum-free medium (12 ± 6% cell death after 3 days of serum-free medium, $n = 4$), whereas mutant PC12 cells derived in parallel (*13*) that retained expression of $p75^{NGFR}$ also retained the characteristic of undergoing cell death in response to serum withdrawal (50 ± 15% cell death after 3 days of serum-free medium, $n = 4$; $P < 0.01$ by paired t test). As an additional control, another plasma membrane protein, β-amyloid precursor protein (β-APP_{751}), was expressed with the pBabe-puro expression vector in the same conditionally immortalized neural cell line (CSM 14.1), without effect on apoptosis (*14*). This does not exclude the possibility that the expression of other proteins may enhance apoptosis.

Although both NGF and mAb directed against $p75^{NGFR}$ enhanced cell survival, and although Trk A is not expressed by CSM 14.1 cells (Fig. 1A), it was possible that NGF inhibited the death of tempera-

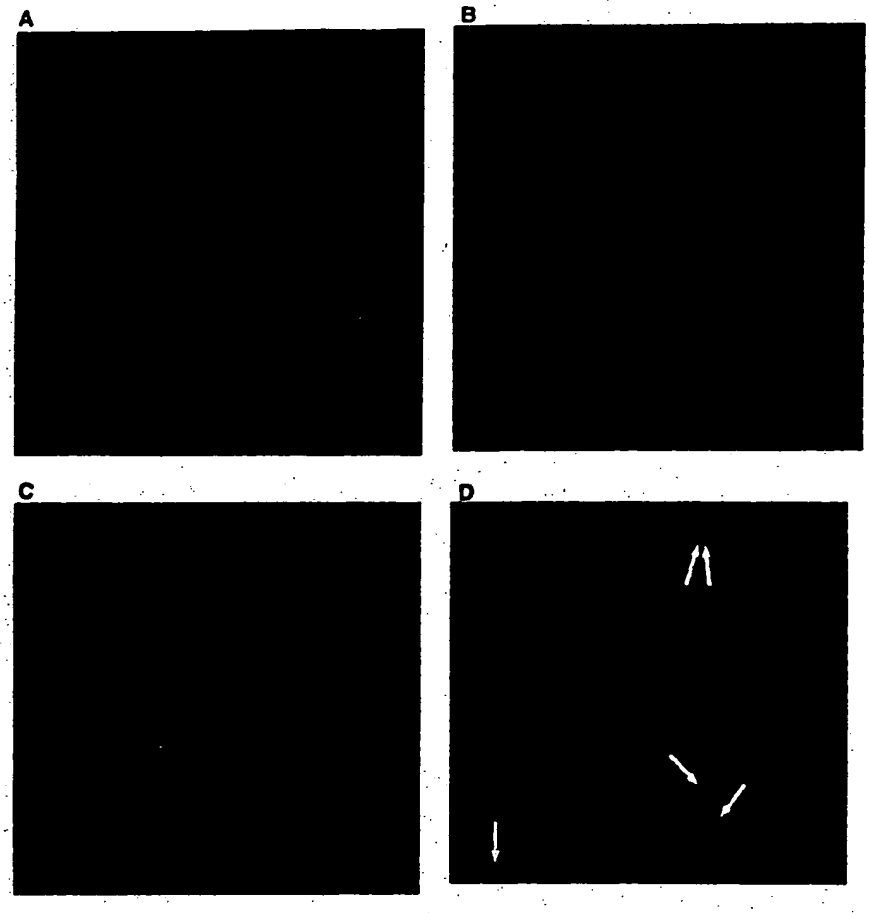

Fig. 3. Apoptosis in R2 cells (*10*) transfected with pBabe-puro-p75^{NGFR}, but not in R2 cells transfected with pBabe-puro. Cells were grown in DMEM with FBS (10%) at 34°C, seeded at a density of 5 × 10^4 cells/cm^2, and placed at 39°C in serum-free medium. After 6 days in serum-free medium, propidium iodide was added at a concentration of 10 μM, and cells were viewed with a Zeiss Axiovert microscope. (A and B) R2 cells transfected with pBabe-puro. (C and D) R2 cells transfected with pBabe-puro-p75^{NGFR}. (A and C) Phase contrast. (B and D) Fluorescence. In (D), many nuclei are fragmented, which is characteristic of apoptosis (single arrows mark some examples); other nuclei are homogeneously stained, also characteristic of apoptosis. The only example of a nonapoptotic nucleus in this field is denoted by a double arrow. Magnification, ×320.

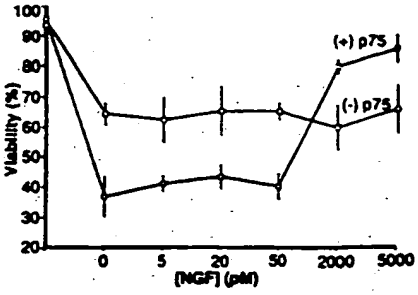

Fig. 4. Inhibition of conditionally immortalized neural cell death by various concentrations of NGF. CSM 14.1 cells were grown as described in Fig. 2. Serum-free medium included the indicated concentrations of NGF. Error bars represent standard deviations (n = 3).

ture-sensitive immortalized neural cells expressing p75^{NGFR} by binding to the high-affinity receptor [dissociation constant (K_d) = 2.3 × 10^{-11} M (*15*)] rather than the low-affinity receptor [K_d = 1.7 × 10^{-9} M (*15*)]. Therefore, several concentrations of NGF were tested. The inhibition of cell death by NGF in this cell line was minimal at concentrations of NGF that bind only the high-affinity NGF receptor significantly (Fig. 4). In contrast, concentrations of NGF equaling or exceeding the affinity constant for binding to the low-affinity receptor increased cell survival (Fig. 4). Survival of control CSM 14.1 cells transfected with the expression construct lacking the p75^{NGFR} open reading frame was not increased by NGF (Fig. 4).

Thus, the expression of p75^{NGFR} resulted in an enhancement of neural cell death in serum-free medium when p75^{NGFR} was not bound by ligand or antibody, whereas enhancement of survival beyond that of controls occurred with binding of the receptor. This dichotomous response defines a previously undescribed type of receptor function within the nervous system. This effect of p75^{NGFR} may account for the fact that some cells become dependent for their survival on the binding of NGF. Early neural cell precursors are independent of NGF, but during development specific neural cells become dependent on NGF (*16*). Increased expression of p75^{NGFR}, which has been shown to occur during development (*17*), could conceivably effect such a switch. Although binding of NGF to Trk A enhances cellular survival and differentiation (*1*), active induction of cell death in the absence of NGF may also occur, and this may be mediated at least in part by p75^{NGFR}. The type of cell death induced by p75^{NGFR}—apoptosis—is the same as that induced by growth factor withdrawal (*18*). However, we cannot exclude the possibility that p75^{NGFR} may under some conditions induce necrosis, especially because the TNFRs may mediate either apoptosis or necrosis (*19*). Our results suggest an additional function for p75^{NGFR} in neural cells, but have no bearing on the other functions ascribed to p75^{NGFR} or on the interaction of other neurotrophins, such as brain-derived neurotrophic factor, with p75^{NGFR}. However, the enhancement of neural cell survival by binding of NGF or mAb to p75^{NGFR} suggests that a similar effect might occur when p75^{NGFR} is bound by other neurotrophins. Neither do the results bear on the role of p75^{NGFR} in the death of non-neuronal cells, such as astrocytes or developing renal cells.

Somewhat similar receptors have been described, including the TNFRs, FAS (Apo-1), and CD40. These molecules show general structural similarity to p75^{NGFR}, with similar extracellular cysteine-rich pseudorepeats and a single transmembrane domain (*20*). The structural similarity of p75^{NGFR} to the other members of the superfamily occurs in the extracellular domain (*5*), but the functional similarity may result from the transduction of a signal leading to (or inhibiting) cell death. The function of p75^{NGFR} is analogous to that of CD40 in that expression occurs on developing cells [mainly central cholinergic, sympathetic, and sensory neurons in the case of p75^{NGFR}, centroblasts and centrocytes in the case of CD40 (*6*)], and leads to a requirement for binding if survival is to occur. In both cases, binding of the receptor leads to improved, but incomplete, cell survival (Figs. 2 and 4) (*6*). Other determinants are clearly involved, because binding of antigen by developing B cells also enhances survival (*6*), lack of expression of CD40 ligand does not result in a reduction

in circulating B cells (*21*), and neural cells expressing $p75^{NGFR}$ survive in media containing serum (Fig. 2). The mechanism by which unbound $p75^{NGFR}$ or other members of this receptor superfamily lead to neural cell death is unknown. However, the structural and functional relation between $p75^{NGFR}$ and TNFR I and II suggests that they may have similar mechanisms of action.

The highest level of expression of $p75^{NGFR}$ in the central nervous system occurs in cholinergic neurons of the nucleus basalis of Meynert, the cells most severely affected in Alzheimer's disease. These cells continue to express normal (*22*) or supranormal (*23*) amounts of $p75^{NGFR}$ mRNA and protein during the neuronal degeneration associated with Alzheimer's disease. In contrast, cholinergic cells of the brainstem that resemble those of the nucleus basalis morphologically, but do not express $p75^{NGFR}$ (*24*), do not degenerate in Alzheimer's disease (*25*).

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13. PC12 cells were maintained in Dulbecco's minimum essential medium (DMEM; Gibco) containing 5% horse serum and 5% supplemented calf serum (Hyclone), in 12% CO_2 at 37°C. Cells were mutagenized with 10 mM ethyl methanesulfonate for 6 hours and then washed with 2 × 10 ml of medium. After growing for 10 days, cells were trypsinized and subcultured into 36 plates at an approximate density of 10^4 cells per plate. The cells were grown for 10 days (approximately five divisions) and then treated with NGF (25 ng/ml) for 5 days. Groups of clonally derived cells that did not respond to NGF by extending neurites were isolated, subcloned, and then tested for the absence of NGF-induced neurite outgrowth and the presence (or absence) of $p75^{NGFR}$ by Northern (RNA) and protein immunoblot analysis. The two subclones used in these experiments were the NRA5 subclone, which does not express $p75^{NGFR}$, and NR5D, which does express $p75^{NGFR}$.
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28. The $p75^{NGFR}$ cDNA in pUC9 was digested with Sal I, filled in with Klenow fragment and deoxynucleotide triphosphates, and then digested with Bgl II. The 1.7-kb fragment containing the entire open reading frame of $p75^{NGFR}$ was then ligated into pUC18 that had been digested with Sma I and Bam HI. The resulting plasmid was digested with Eco 47III and Sal I and ligated into pBabe-puro (*8*) that had been cut with Sna BI and Sal I, to create pBabe-puro-$p75^{NGFR}$. CSM 14.1 cells (*7*) are rat nigral neural cells immortalized with the temperature-sensitive large T antigen of SV40. These cells express tyrosine hydroxylase, neuron-specific enolase, and neurofilament (NF-L). CSM 14.1 cells were transfected with pBabe-puro-$p75^{NGFR}$ with the cationic lipid DOTAP (Boehringer Mannheim, Inc.) and then selected in puromycin (7 μg/ml). The comparison of single colonies can introduce bias into the results (*9*), but this was obviated by comparison of entire pools of stable transfectants (*9*); therefore, pools of stable transfectants (populations including more than 100 separate colonies) with pBabe-puro-$p75^{NGFR}$ were compared with pools of pBabe-puro transfectants. Cells were grown in DMEM with fetal bovine serum (FBS) (10%) at 34°C in 5% CO_2. Total RNA was prepared by the method of Chomczynski (*26*), and electrophoresis was carried out in formaldehyde gels. After Northern transfer to nylon, ^{32}P-labeled probes for $p75^{NGFR}$ (1-kb cDNA fragment, digested with Stu I), Trk A (0.5-kb cDNA fragment, digested with Xho I), and γ-actin were hybridized sequentially. Blots were exposed to film for 24 hours for the $p75^{NGFR}$ and Trk A probes and for 2 hours for the γ-actin probe. For immunocytochemistry, cells were fixed in paraformaldehyde (4%) for 15 min and permeabilized in 0.1% Triton X-100. Immunocytochemistry was done as described (*27*), with a polyclonal antibody (1:2500) to purified $p75^{NGFR}$. As controls, primary antibody was omitted and control transfectants were stained; both of these controls showed a similar lack of staining.
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Redundant Mechanisms of Calcium-Induced Calcium Release Underlying Calcium Waves During Fertilization of Sea Urchin Eggs

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Propagating Ca^{2+} waves are a characteristic feature of Ca^{2+}-linked signal transduction pathways. Intracellular Ca^{2+} waves are formed by regenerative stimulation of Ca^{2+} release from intracellular stores by Ca^{2+} itself. Mechanisms that rely on either inositol trisphosphate or ryanodine receptor channels have been proposed to account for Ca^{2+} waves in various cell types. Both channel types contributed to the Ca^{2+} wave during fertilization of sea urchin eggs. Alternative mechanisms of Ca^{2+} release imply redundancy but may also allow for modulation and diversity in the generation of Ca^{2+} waves.

Transient increases in the concentration of calcium ions ($[Ca^{2+}]_i$) act as cell signals. In general, the signal shows spatial and temporal inhomogeneity and takes the form of waves or oscillations within the cell (*1*). Several mechanisms have been proposed to account for regenerative Ca^{2+} release (*2*). Release of Ca^{2+} from internal stores can be stimulated by an increase in $[Ca^{2+}]_i$; this process is termed Ca^{2+}-induced Ca^{2+} release (CICR) (*3*). This Ca^{2+} release appears to be mediated by Ca^{2+} channels in the endoplasmic reticulum (ER) that are sensitive to cytoplasmic agonists, to $[Ca^{2+}]_i$, and to the amount of Ca^{2+} in the lumen of the ER (*4*). Two closely related Ca^{2+} channels with these properties are the inositol trisphosphate (IP_3) receptor (IP_3R) (*5*) and the

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A region of the 75 kDa neurotrophin receptor homologous to the death domains of TNFR-I and Fas

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Abstract Members of the NTR/TNFR family mediate apoptosis in many tissues, yet sequence homology has not been detected in their intracellular domains except for a 'death domain' in TNFR-I and Fas. Here, a region of the 75 kDa neurotrophin receptor (NTR) has been aligned with this apoptosis-inducing motif. Peptides at the carboxyl terminus of each domain potentially form amphiphilic helices, one of which (in NTR) resembles mastoparan, a G-protein activating peptide. Molecular models of these death-region peptides suggest that observed sequence similarities reflect a common structure, perhaps capable of undergoing an induced coil to helix transition.

Key words: Neurotrophin receptor; NGF receptor; Molecular modeling; Apoptosis; Alpha-helix; Mastoparan

1. Introduction

Receptors of the NTR/TNFR family function during development and in adult tissues by regulating apoptotic cell death [1,2]. All possess extracellular domains composed of repeating units that fold into a characteristic structure [3,4]. However, except for an 'apoptosis motif' in TNFR-I (55 kDa) and Fas/Apo1, neither sequence nor structural similarity has been detected in the intracellular domains of these molecules [5–7].

Various signaling mechanisms have been proposed for members of the family. Although direct substrate phosphorylation has been ruled out by the absence of catalytic domains, interactions with cytoplasmic signaling molecules apparently link activated receptors to signaling cascades [8]. The established function of the neurotrophin receptor (NTR) is to modulate the affinity and activity of tyrosine kinases that promote neuronal survival [9]. It was recently shown, however, to induce apoptotic cell death much like that stimulated by TNFR-I and Fas [10,11]. Unlike TNFR-I and Fas, cell death induced by NTR is reversed rather than caused by ligand binding. This function is consistent with observations that its contribution to the survival response can best be measured at high ratios of NTR to coexpressed trk receptors, which also bind neurotrophins [9]. These reports prompted a re-examination of the intracellular domain of NTR for possible similarities to TNFR-I and Fas, which may signal through a related mechanism, i.e. sphingomyelinase activation [12–14].

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Abbreviations: NTR, 75 kDa neurotrophin receptor; TNFR, tumor necrosis factor receptor; GDP, guanosine diphosphate; G-protein, heterotrimeric guanine nucleotide-binding regulatory protein.

Here, primary sequence alignment was used to locate a death domain in the intracellular portion of the neurotrophin receptor. To explore structural implications of this homology, putative structures were modeled using computer-aided molecular design and visualization tools. Peptides in the death-mediating region appear to be structurally similar to peptides that undergo a transition from coil to helix, and which activate heterotrimeric G-proteins.

2. Experimental

2.1. Alignment and analysis of secondary structure

Amino acid sequences having the most complete annotation were obtained from databases as follows: SwissProtein; human fas antigen – P25445, chicken NTR – P18519, and rat TNFR-I – P22934. PIR; mouse fas antigen – A46484 and human TNFR-I – A34899. GenBank; rat fas antigen – D26112, human NTR – M14764, rat NTR – X05137 and mouse TNFR-I – M59378. Sequences and measurements of biological activity were obtained for insect venom peptides as follows: MP, MP-X, MP-A', MP-T, Mas7, Mas9 and Mas19 [15]; MP14 and MP15 [16]; melittin [17]. Matrix calculations were performed by DNA Strider version 1.2 (Christian Marck, CEN-Saclay, France).

Initial alignments were prepared using the algorithm of Altschul [18]. Multiple alignments were optimized by hand using SeqVu version 1.0.1 (James Gardner, Garvan Institute of Medical Research, Sydney, Australia), based on sidechain chemistry and rational placement of gaps. The methods of Garnier et al. and Chou and Fasman, as modified by Nishikawa [19], were to evaluate the probability that a sequence could adopt a helical structure. Helical wheels were drawn as described by Schiffer and Edmundson [20].

2.2. Model building

Hypothetical structures were assembled SYBYL version 6.1 (Tripos Associates, St. Louis, MO) on Silicon Graphics workstations. Free amino and carboxylate groups were left at the ends. Van der Waals conflicts resulting from sidechain substitutions were resolved by rotamer library and molecular mechanics functions in SYBYL. Energy minimization was carried out using the AMBER all-atom force field, a distance-dependent dielectric constant of 4.0 R and a non-bonded atom cutoff of 10 Å. Computer graphics were prepared with MIDASPlus [21] and displayed using the conic delegate [22].

3. Results

3.1. Identification of death-domain homology in NTR

Because widely-used alignment algorithms had not detected similarity in the intracellular domains of the NTR/TNFR family, representative sequences were evaluated with a matrix procedure. This method looks for matches (amino acid identities) within a sliding window. The test is relatively insensitive to gaps, and can be adjusted to detect low levels of amino acid identity. Comparisons of NTR with TNFR-I and Fas intracellular domains detected matches in the previously-identified death domain at approximately 27% sequence identity.

Using short, matched sequences from the matrix and homologs from additional species, residues 330 to 390 of NTR were

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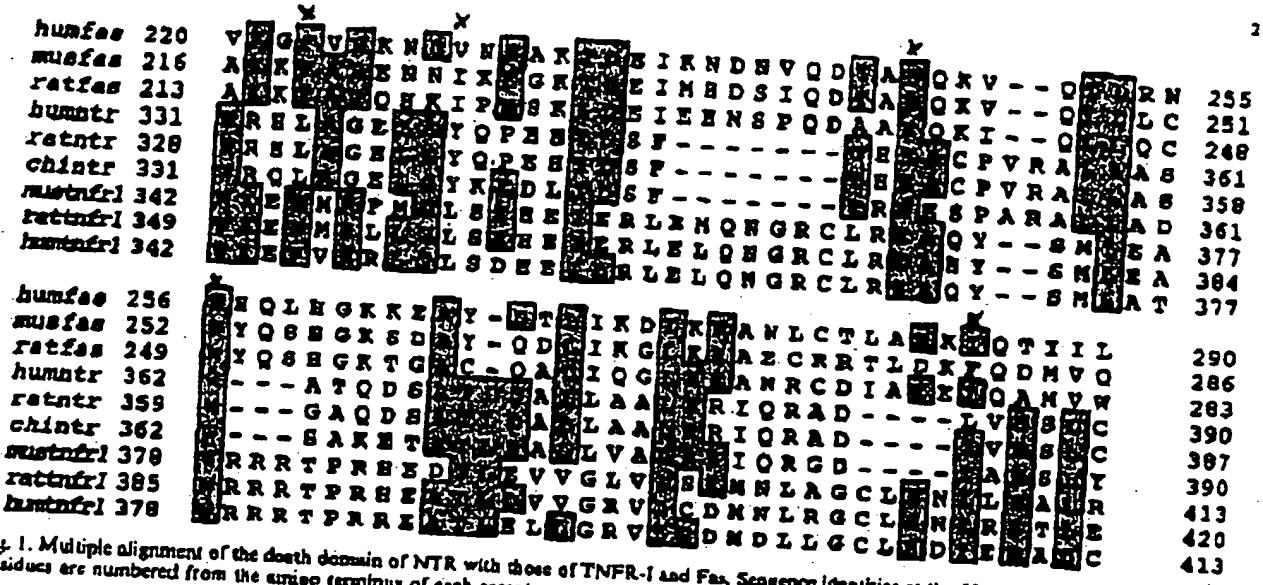


Fig. 1. Multiple alignment of the death domain of NTR with those of TNFR-1 and Fas. Sequence identities at the 50% level are boxed and shaded. Residues are numbered from the amino terminus of each receptor.

aligned with the death regions of TNFR-1 and Fas (Fig. 1). The alignment begins near the amino terminus of the apoptosis domain identified by Itoh et al. [5], and extends through the region probed by alanine-scanning mutagenesis of TNFR-1 [6]. The arrangement of the domain suggests that it may have arisen as a duplication of approximately 38 codons [23]. While NTR and TNFR-1 share higher sequence identity (32.8%), TNFR-1 and Fas (25.4% identity) are more closely related with respect to the placement of gaps and insertions. Several structural motifs appear to be anchored by highly-conserved residues. The functions of Glu-369, Trp-378 and Ile-408 were tested by mutation to Ala in TNFR-1 [6]. Substitution of these residues, conserved in the homology with NTR, blocked transmission of apoptotic signals.

3.2. Potential for formation of helical structure

Unexpected patterns of sequence gaps and amino acid identities were investigated first by evaluating secondary-structure propensities in the aligned domain. Two algorithms [19] independently predicted alpha-helical conformations for most residues of the nine sequences aligned in Fig. 1. For the human homologs of Fas, TNFR-1 and NTR, regions are plotted in Fig. 2A in which both methods predict alpha helices. Fas and TNFR-1 show helical potential in the amino-terminal half of the alignment. These segments may engage in self-association as a consequence of activation by ligand, since deletions that eliminate dimerization and block signaling have been mapped to this region [24,25].

Common to all three receptors is a potential helix in the carboxyl-terminal half of the aligned region (Fig. 2A). This sequence in NTR was previously identified as a homolog of the wasp venom peptide, mastoparan [26]. To determine whether the sequences of TNFR-1 and Fas might also encode mastoparan-like helices, peptides from the shared part of this segment were analyzed by display in helical-wheel plots (Fig. 2B). The arrangement of sidechains strongly suggests the possibility of forming amphiphilic structures having one hydrophobic surface and one electrostatically-charged surface. Potential of this region to form an amphiphilic helix has been recognized for NTR and TNFR-1 [26,27], but not for Fas.

3.3. Sequences of three receptors can be modeled as stable, low-energy helices

Alignment (Fig. 1) and secondary-structure prediction (Fig. 2) pointed to potential structural similarities among the three receptors. To visualize these structures, hypothetical models were constructed using molecular design software. Residues 245–284 of human Fas (40 monomers) were modeled with alpha-helical phi and psi angles, then energy minimized using molecular mechanics. The AMBER force field was used to estimate the total energy of the model after minimization, calculated to be −188.7 kcal/mol. Models representing human NTR residues 349–384 (36 monomers) and human TNFR-1 residues 367–407 (41 monomers) were similarly prepared. These minimized respectively to −172.9 kcal/mol and −223.5 kcal/mol.

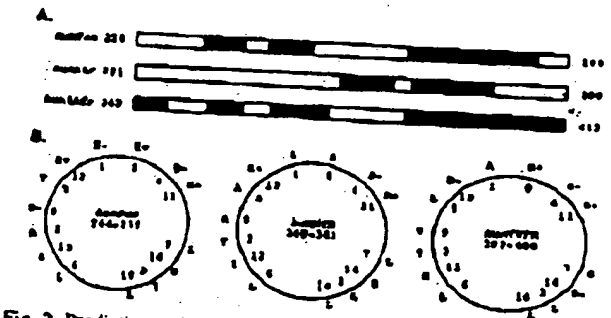


Fig. 2. Predictions of secondary structure in the region of homology. (A) Aligned sequences are represented as open bars. Consensus segments of alpha helix are plotted in black. (B) Helical wheel representations of the carboxyl-terminal peptides. These show the distribution of amino acid sidechains as seen looking down the axis of the helix from the amino-terminal end.

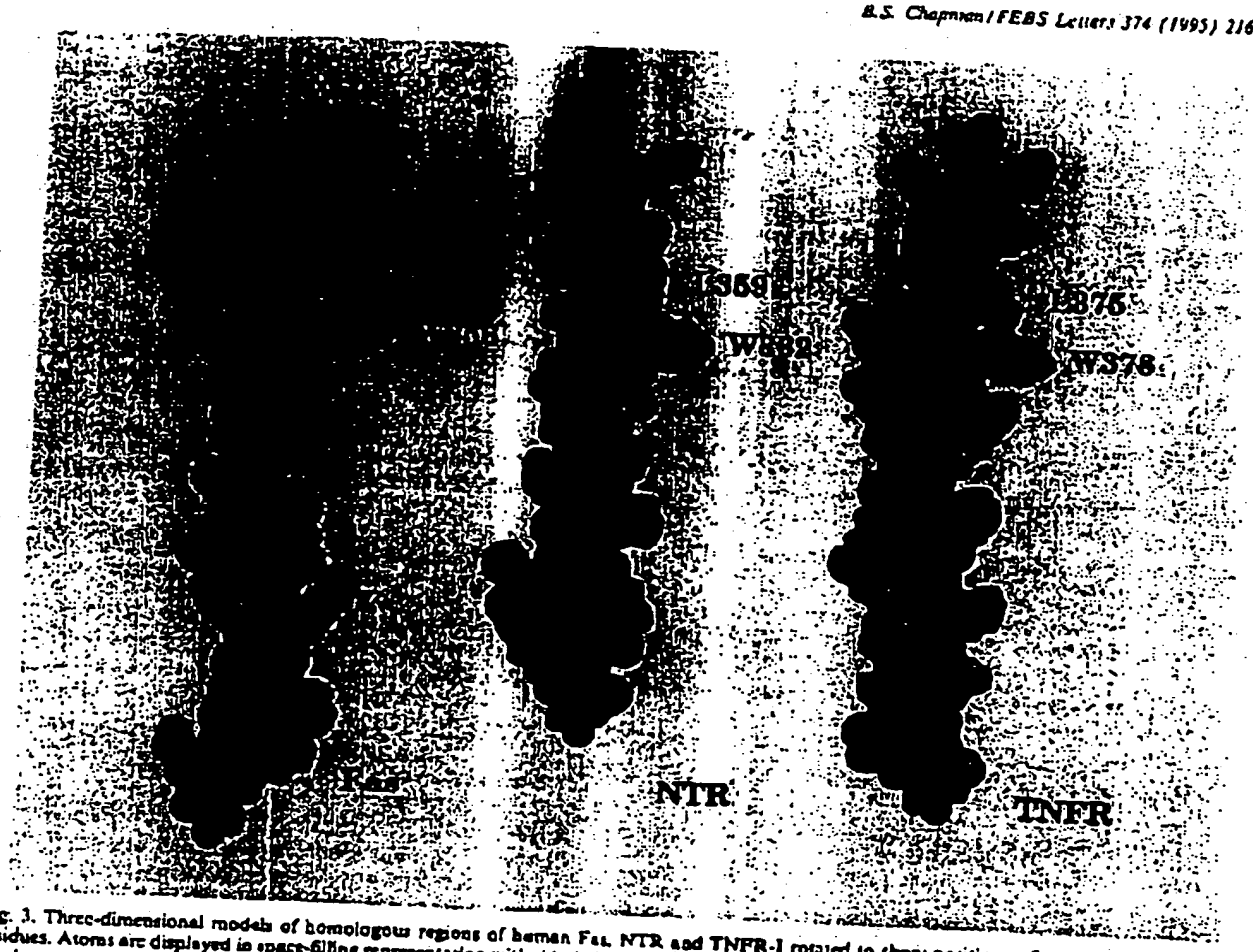


Fig. 3. Three-dimensional models of homologous regions of human Fas, NTR and TNFR-I rotated to show positions of conserved Leu and Trp residues. Atoms are displayed in space-filling representation with sidechains color coded as follows: polar, dark gray; charged, light gray; hydrophobic, black.

Low energies achieved for the models indicate stabilization by extensive hydrogen bonding and excellent van der Waals interactions. The structures are completely theoretical, since neither solvent molecules nor dissolved ions were considered in the calculations. Views of these models are shown in Figs. 3 and 4.

The three-dimensional models start at the end of the first gap in NTR (see Fig. 1), which corresponds to the C-terminal boundary (TNFR-I Leu-367) of the postulated dimerization motif [25]. Fig. 3 shows the prominent feature created near the amino terminus of the helix by sidechains of conserved Leu and Trp residues. The opposite sides of these structures appear different from each other, consistent with experimental results showing that mutation of Trp-378 in human TNFR-I abrogated induction of cell death, but mutation of Arg-379 had no detectable effect [6].

When rotated to display a predicted helical region near its C-terminus, each receptor model presents a striking hydrophobic face (Fig. 4). The aromatic ring of Tyr-266 in Fas appears to substitute for conserved leucines in NTR and TNFR-I, orienting to contribute part of a non-polar surface. A basic sidechain following the last conserved Leu seems to occupy a similar position in each structure (not labeled, coming out of the plane of the figure. See also 2B, where a positively-charged sidechain is indicated at position 11 of each helical wheel). The three receptor models demonstrate structural similarities, despite the low level of sequence identity.

3.4. Potential amphiphilic helices resemble a modeled structure of mastoparan

Naturally-occurring mastoparans and synthetic derivatives have been intensively studied as potent activators of heterotrimeric G proteins [15]. These peptides reportedly assemble into biologically-active helices upon interaction with phospholipid membranes or in the presence of chaotropic salts [17,28]. To investigate resemblances between modeled receptors and mastoparan, a three-dimensional representation was constructed for the fourteen-residue peptide. The mastoparan model relaxed to a total energy of −45.5 kcal/mol, and closely matched the membrane-bound structure of mastoparan-X obtained by 2D-NMR [28]. Like MP-X, modeled mastoparan (Fig. 5) forms an amphiphilic helix in which five aliphatic sidechains contribute to a non-polar surface [28]. This surface has several features in common with the non-polar surfaces of the receptor models (Fig. 4). The basic surface of mastoparan

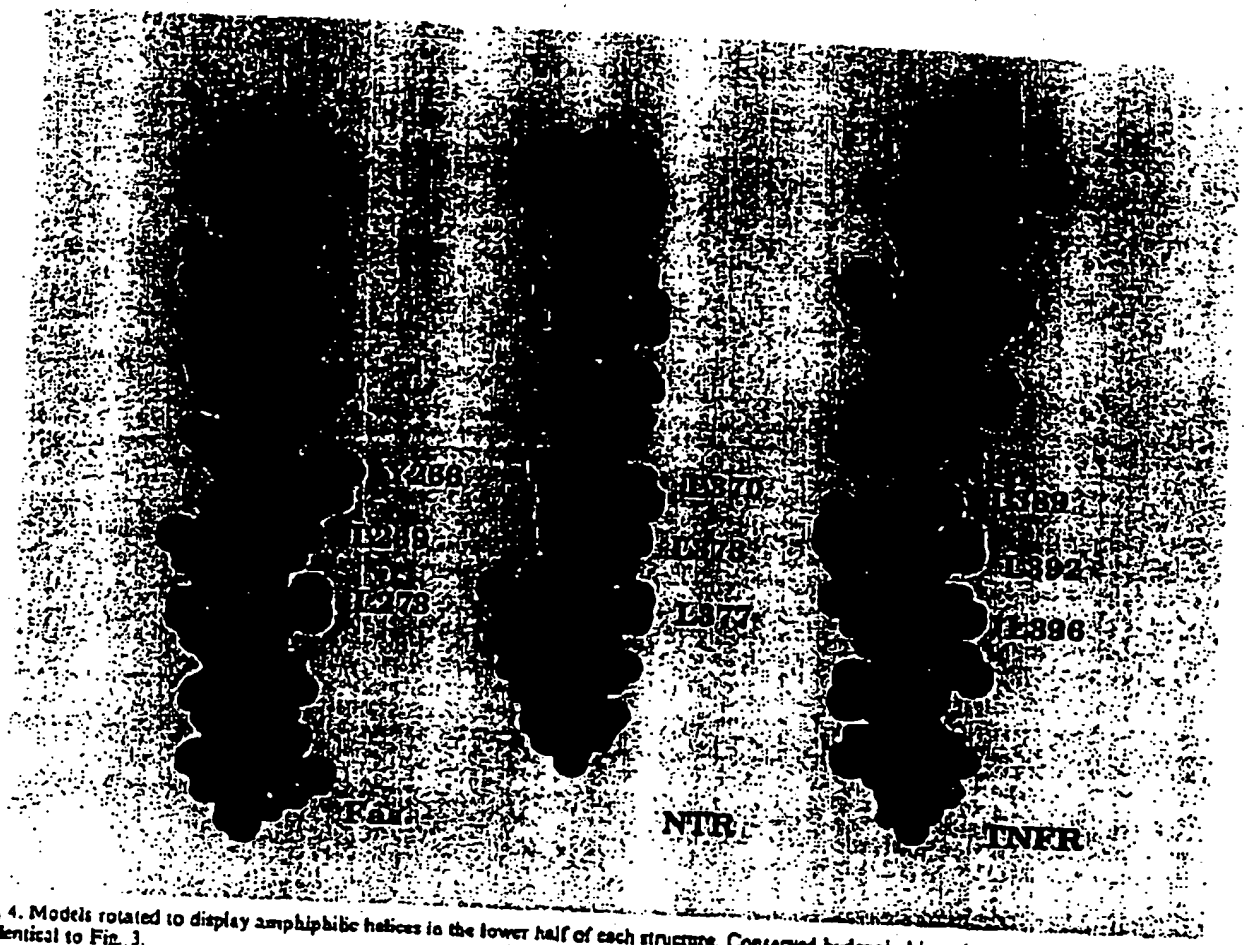


Fig. 4. Models rotated to display amphiphilic helices in the lower half of each structure. Conserved hydrophobic residues are labeled. Color coding is identical to Fig. 3.

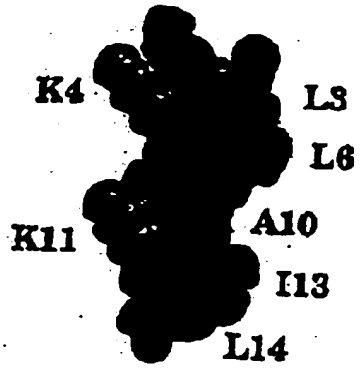
likewise looks like the polar surfaces of the modeled receptors. For example, *Lys*-11 is oriented similarly to the basic sidechain at the bottom of each receptor's hydrophobic face. Modeling thus shows that the death-domain peptides could mimic the mastoparan structure were they to adopt a helical conformation.

Mastoparans, biologically-active analogs and bee-venom peptide (melittin) undergo a conformational transition from coil to helix under specific conditions [17,28]. Amino acid substitutions in these peptides can produce molecules that neither stimulate G-proteins nor form helices [15,17,28]. To evaluate chemical similarities at critical positions, death-domain sequences containing putative amphiphilic helices were aligned with biologically-active mastoparans and melittin (Fig. 6). The three apoptosis-motif peptides show strict conservation of aliphatic residues at mastoparan positions 2, 6, 10 and 13. All other positions display substitutions comparable to at least one active molecule, except for the fourteenth residue, which is an amide or acid in the receptor peptides and aliphatic in the insect peptides. Chemical similarity between active mastoparans and segments of TNFR-1, Fas and NTR suggests these death-domain peptides may be able to undergo an induced coil to helix transition.

4. Discussion

Standard methods for detecting homology have consistently failed to find sequence similarities in intracellular domains of the NTR/TNFR family. This has led to the suggestion that these receptors may be chimeras [7]. An alternative possibility is that each member has undergone extensive evolution from a common ancestor in the process of adapting its structure for specific, non-catalytic functions. Shown here are significant evolutionary and structural relationships in the intracellular domains of the neurotrophin receptor, Fas antigen and the 55 kDa TNF receptor. The death domain in NTR appears to be a robust homolog, since the percent identity with TNFR-1 is higher than between TNFR-1 and Fas, and because several residues conserved in the alignment are known to be critical for function [6].

Death-domain signaling has been difficult to unravel, owing to its novel structure and complex regulation. While protein phosphorylation may be important, experiments have not clarified whether kinases and phosphatases are primarily used for signaling or for regulation. In identifying a mastoparan-like peptide in the C-terminal half of each apoptosis domain, the present study expands the list of potential mechanisms. If con-



Mastoparan

Fig. 5. Hypothetical model of mastoparan. This structure has a free carboxylate at the C-terminus, differing slightly from mastoparans and melittin, which are amidated. Color coding of sidechains is the same as in Fig. 3.

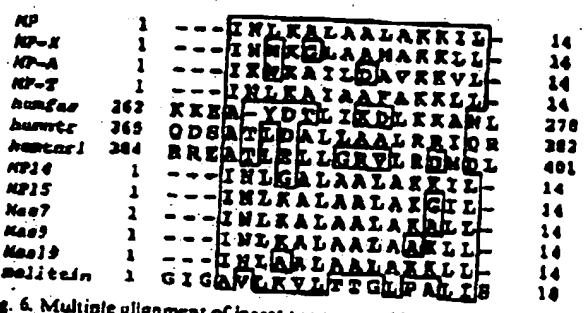


Fig. 6. Multiple alignment of insect venom peptides and synthetic analogs with potential amphiphilic helix residues of human Fas, TNFR-I and NTR. Residues chemically homologous to the mastoparan sequence are boxed (e.g. aliphatic, aromatic, amidated, hydroxylated, basic, acidic).

formational change from coil to helix were induced by receptor activation, such a peptide would act as a switch. A peptide representing NTR residues 367–379 has been shown by circular dichroism spectroscopy to assume a helical conformation in non-polar solvent [27]. The potentially amphiphilic peptides of TNFR-I and Fas should also be tested for ability to undergo the coil to helix transition. NTR, when activated, has been shown to stimulate Gi proteins in isolated membranes [29]. It may be worth exploring the pertussis-toxin sensitivity of TNFR-I and Fas signaling.

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